Scientific Committee on Emerging and Newly Identified Health Risks

SCENIHR

Opinion on the

# Guidance on the Determination of Potential Health Effects of Nanomaterials Used in Medical Devices

SCENIHR adopted this opinion by written procedure on 6 January 2015

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http://ec.europa.eu/health/scientific_committees/emerging/members_wg/index_en.htm

## ABSTRACT

This Guidance addresses the use of nanomaterials in medical devices and provides information for risk assessors regarding specific aspects that need to be considered in the safety evaluation of nanomaterials. According to the EU Recommendation for the definition of a nanomaterial (Commission Recommendation 2011/696/EU, EC 2011) any particulate substance with at least one dimension in the size range between 1 and 100 nm is considered a nanomaterial. These particles (nanoparticles) exhibit specific characteristics that differ from the characteristics of larger sized particles with the same chemical composition.

The use of nanomaterials in medical devices poses a challenge for the safety evaluation and risk assessment of these medical devices as the specific character of the nanomaterial used should be taken into consideration. The various aspects of safety evaluation and risk assessment of medical devices containing nanomaterials are addressed in this Guidance. The use of nanomaterials in medical devices can vary considerably. Examples are the use of free nanomaterials being a medical device and administered to the patient as such (e.g. iron oxide or gold nanomaterials for heat therapy against cancer), free nanomaterials in a paste-like formulation (e.g. dental filling composites), free nanomaterials added to a medical device (e.g. nanosilver as antibacterial agent in wound dressings), fixed nanomaterials forming a coating on implants to increase biocompatibility (e.g. nano-hydroxyapatite) or to prevent infection (e.g. nano-silver), or embedded nanomaterials to strengthen biomaterials (e.g. carbon nanotubes in a catheter wall). In all these cases the potential exposure to the nanomaterials should be considered. It is additionally recognised that wear-and-tear of medical devices may result in the generation of nanosized particles even when the medical device itself does not contain nanomaterials.

Guidance is provided on physico-chemical characterisation of nanomaterials, the determination of hazards associated with the use of nanomaterials, and risk assessment for the use of nanomaterials in medical devices. The safety evaluation of the nanomaterials used in medical devices is discussed in the context of the general framework for biological evaluation of medical devices as described in the ISO 10993-1:2009 standard. Therefore, the risk assessment should be performed taking into consideration the type of device, the type of tissue contact, and the contact duration, thus identifying the specific exposure scenario.

This Guidance provides information to assist with safety evaluation and risk assessment on the use of nanomaterials in medical devices that should be considered in conjunction with the ISO 10993-1:2009 standard. The Guidance highlights the need for special considerations in relation to the safety evaluation of nanomaterials, in view of the possible distinct properties, interactions, and/or effects that may differ from conventional forms of the same materials. For some assays evaluating potential hazards of nanomaterials adaptation of existing assays may be necessary. In addition, it is also possible to apply this Guidance for the safety evaluation and risk assessment of particles with a size larger than 100 nm.

A phased approach is recommended for evaluating the risk of the use of nanomaterials in medical devices based on potential release and characteristics of the nanomaterials to avoid unnecessary testing. The phases cover particle release (phase 1), particle distribution and persistence (phase 2), hazard assessment (toxicological evaluations) (phase 3), risk characterisation/risk assessment (phase 4). In phase 1 an evaluation of the potential for the device to release nanoparticles either directly or due to wear of the device during use should be carried out. In phase 2 the aim is to determine the distribution of the particles released and also their persistence potential. In phase 3 the hazard is assessed using appropriate toxicity tests taking account of the exposure characteristics and potential for persistence in specific organs. This will provide input for the final risk characterisation (phase 4). The estimated risk needs to be compared to the

risk from the use of comparable devices not incorporating nanomaterials in judging the acceptability of the risk.

In conclusion, the potential risk from the use of nanomaterials in medical devices is mainly associated with the possibility for release of free nanoparticles from the device and the duration of exposure.

**Keywords**: Medical devices, nanomaterials, risk evaluation, SCENIHR, Scientific Committee on Emerging and Newly Identified Health Risks.

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## 1. BACKGROUND

Today, nanotechnologies and nanomaterials are being used more widely, or will soon be used, in a variety of fields including health care. For nanomedicine, the three largest areas of application are diagnostics, drug delivery and regenerative medicine (ETP Nanomedicine 2009). In addition there are applications in surgery and thermotherapy (Vauthier *et al.* 2011).

In the field of medical devices, the alleged use of nanomaterials has been identified by Notified Bodies in the following applications:

- Carbon nanotubes in bone cements;
- Nanopaste hydroyapatite powder for bone void filling;
- Polymer setting material with nanoparticles in dental cements;
- Polycrystalline nanoceramics in dental restorative materials;
- Nanosilver or other nanomaterials used as coatings on implants and catheters;
- Nanosilver used as an antibacterial agent, for example in wound dressings (see also Wijnhoven *et al.* 2009).

Furthermore, there are reports of iron-oxide nanoparticles being injected into tumour cells to be heated-up by radiation or an external magnetic field[1]**.** It is not clear if this type of use falls under the legislation on medicines or the legislation on medical devices. On one hand, the immediate effect is mechanical because the tumour cells burst. On the other hand, the legislation on medicines might be applicable because the burst cells are later metabolised.

Although the general risk assessment requirements applicable for materials used in medical devices and previous scientific opinions on risk assessment of nanomaterials (see e.g. SCENIHR 2006, 2007 and 2009) are useful when assessing nanomaterials for medical applications, further clarification is needed in the risk assessment of such products. This is particularly true for medical devices, considering the decentralised regulatory system ("New Approach"). The risk assessor, be it the manufacturer, the Notified Body or the authority, should be aware of the specific characteristics of nanomaterials in order to do the risk assessment of the application of nanomaterials in a medical technology.

The European Commission has published two proposals for revision of the medical devices legislation: a Proposal on medical devices (COM(2012)542) and a Proposal on *in vitro* diagnostic medical devices (COM(2012)541). These proposals include a definition of nanomaterial taken from Commission Recommendation 2011/696/EU on the definition of nanomaterial and provisions on the risk classification, the labeling and the instructions for use of medical devices containing nanomaterial. In addition, the general safety and performance requirements now contain a specific requirement to design and manufacture medical devices in such a way as to reduce to a minimum the risks linked to the size and the properties of particles used. Special care should be applied when devices contain or consist of nanomaterial that can be released into the patient's or user's body. The risk classification influences the stringency of the applicable conformity assessment procedure.

[1] See as an example for the latter the product description of MagForce at: http://www.magforce.de/english/home1.html

## 2. TERMS OF REFERENCE

In light of the expected increase in the application of nanotechnologies to medical devices, the SCENIHR is requested to provide a Guidance on the risk assessment of medical devices containing nanomaterials. This Guidance should enable the classification of different categories of medical devices containing nanomaterials according to their level of risk.

This Guidance should take into account different categories of medical devices such as:

a. Non-invasive medical devices, e.g. devices coming into contact with the intact skin,

b. Invasive devices (surgical or not), e.g.:

- Wound care materials,
- implantable medical devices,
- dental and bone fillings and cements,
- injectable nanomaterials.

In this assessment, where relevant, the SCENIHR is invited to differentiate between free, fixed, and embedded nanomaterials.

The Guidance should also differentiate the cases where the nanomaterial might inadvertently be released into the patient's or user's body and the cases where the nanomaterial is deliberately intended to be released into the human body.
Deadline: December 2013

Supporting documents:

Afssaps (Agence française de sécurité sanitaire des produits de santé), Biological assessment of medical devices containing nanomaterials – Scientific Report (19.8.2011).[2]

ETP Nanomedicine (2009). Roadmaps in nanomedicine towards 2020. Downloadable from http://www.etp-nanomedicine.eu/public/press-documents/publications/etpn-publications

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[2] http://www.afssaps.fr/Activites/Surveillance-du-marche-des-dispositifs-medicaux-et-dispositifs-medicaux-de-diagnostic-in-vitro-DM-DMDIV/Dispositifs-medicaux-Operations-d-evaluation-et-de-controle-du-marche/Dispositifs-medicaux-Operations-d-evaluation-et-de-controle/Evaluation-biologique-des-dispositifs-medicaux-contenant-des-nanomateriaux

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# 3. GUIDANCE ON SAFETY EVALUATION OF NANOMATERIALS USED IN MEDICAL DEVICES

## 3.1. Introduction

Nanomedicine is one of the most promising fields of application of nanotechnologies. It uses new physical, chemical and biological properties related to nanoscale structures in medicinal products and medical devices, which provide opportunities, but may also be associated with risks.

This Guidance focuses specifically on medical devices. The directive 93/42/EEC as amended by Directive 2007/47/EC defines a medical device as "any instrument, apparatus, appliance, software, material or other article, whether used alone or in combination, including the software intended by its manufacturer to be used specifically for diagnostic and/or therapeutic purposes, and necessary for its proper application, intended by the manufacturer to be used for human beings for the purpose of:

- diagnosis, prevention, monitoring, treatment or alleviation of disease,
- diagnosis, monitoring, treatment, alleviation of or compensation for an injury or handicap,
- investigation, replacement or modification of the anatomy or of a physiological process,
- control of conception,

and which does not achieve its principal intended action in or on the human body by pharmacological, immunological or metabolic means, but which may be assisted in its function by such means."

This definition is slightly amended in the proposal for a new Medical Device Regulation currently (2014) under negotiation (EC 2012). The proposed changes in the definition do not impact this Guidance.

It should be noted that devices might incorporate a substance as an integral part, which if used separately, might be considered to be a medicinal product. The safety, quality and usefulness of the medicinal substance must be verified by analogy with the methods required by in Directive 2001/83/EC (Medicinal Products for Human Use) concerning the testing of medicinal products.

The proposal for a new Medical Device Regulation includes a definition of nanomaterials and provisions on the risk classification, the labeling and the instructions for use of medical devices containing a nanomaterial. In addition, the general safety and performance requirements in the proposal contain a specific requirement to design and manufacture medical devices to minimise the risks linked to the size and the properties of particles used, whereby special care should be applied when devices contain or consist of a nanomaterial that can be released into the patient's or user's body. The proposal designates medical devices containing nanomaterials in the highest risk class (class III) because of the uncertainties still associated with the potential risks of nanomaterials.

The use of nanomaterials in medical devices can vary considerably. Examples are the use of free nanomaterials as a medical device which is administered to the patient (e.g. iron oxide or gold nanomaterials for heat therapy against cancer), free nanomaterials in a paste-like formulation (e.g. dental filling composites), free nanomaterials added to a medical device (e.g. nanosilver as antibacterial agent in wound dressings), fixed nanomaterials forming a coating on implants to increase biocompatibility (e.g. nano-hydroxyapatite) or to prevent infection (e.g. nano-silver), or embedded nanomaterials to strengthen biomaterials (e.g. carbon nanotubes in a catheter wall). In all these cases the potential exposure to the nanomaterials should be considered. It is additionally

recognised that wear-and-tear of medical devices may generate nanosized particles even when the medical device itself does not contain nanomaterials (Gill *et al.* 2012).

In the harmonised European standard ISO 10993-1 "Biological evaluation of medical devices Part 1 Evaluation and testing within a risk management process", general considerations are included on how to perform a biological safety evaluation of medical devices depending on the application and use of the medical device. The following aspects are considered:

- Category of device: surface device, external communicating device, implant device.
- Location of tissue contact: skin, mucosal membrane, breached or compromised surface, blood, tissue, bone, dentin.
- Contact time: defined as, limited ≤24 hours, prolonged > 24 hours to 30 days, permanent >30 days.

Depending on the use of the medical device, a range of tests must be considered for the biological safety evaluation (ISO 10993-1:2009). Subsequent parts of the ISO 10993 series describe more specific aspects or test methods. A Guidance on nanomaterials is currently being prepared, entitled ISO/TR 10993-22 Biological evaluation of medical devices - Part 22: Guidance on nanomaterials.

The nano-related risk of medical devices containing nanomaterials is mainly associated with the possibility of the release of free nanoparticles from the device, and their potential toxic effects. However, toxic effects of fixed nanomaterials due to their chemical composition and/or enhanced reactivity should be included. For this purpose, a detailed characterisation and identification of the nanomaterials is essential.

The safety evaluation and risk assessment of nanomaterials differ from those carried out for conventional substances and pose substantial challenges (SCENIHR 2006, 2007). This Guidance provides information performing risk assessment of medical devices containing nanomaterials, but does not address the risk assessment of particular individual medical devices containing nanomaterials. This risk assessment should be performed on a case-by-case basis, for each specific nanomaterial-containing medical device. Thus, extrapolation from one nanomaterial to another is not possible. For example, nanosilver has intrinsic properties that are quite different from the properties of gold nanoparticles and the properties of 20 nm nanosilver differ from 100 nm nanosilver particles (Park *et al.* 2011).

This Guidance is limited to the use of nanomaterials in medical devices and the risks for patients and users of these devices, i.e. health care professionals and other individuals taking care of a patient and consequently coming into contact with nanomaterials in medical devices. It may apply to nanoparticles generated from wear-and-tear of medical devices not containing nanomaterials. However, it does not address:

- broader topics of application of nanotechnologies in medical devices including e.g. nano-electronics and lab-on-a-chip technologies. Nanotechnologies are enabling technologies with very broad application. Importantly, there are great differences, for example, in risk profiles between applications using nano-electronics – even if they are applied in implants - and applications using nanomaterials.
- *in vitro* diagnostic (IVD) medical devices, because the exposure to the nanomaterials within IVDs is highly unlikely due to the nature of these products.
- medical imaging technologies using contrast agents, because medical imaging equipment is classified as medical devices and contrast agents, which may include or consist of nanomaterials are medicinal products.
- occupational and environmental risks during the manufacturing and disposal of a nanomaterial-containing medical device.

The Guidance addresses the use of nanomaterials as defined in the recommendation of the European Commission of October 2011 (Commission Recommendation 2011/696/EU) (EC 2011), which is also used in the proposed regulation on Medical Devices. Chapter I Scope and Definitions of the proposed Regulation on Medical devices contains Article 2 (15) defining a nanomaterial as follows:

*"nanomaterial' means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1-100 nm.*

*Fullerenes, graphene flakes and single-wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials.*

*For the purposes of the definition of nanomaterial, 'particle', 'agglomerate' and 'aggregate' are defined as follows:*

- *'particle' means a minute piece of matter with defined physical boundaries;*
- *'agglomerate' means a collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components;*
- *'aggregate' means a particle comprising of strongly bound or fused particles".*

Notably, the Commission Recommendation for a nanomaterial (EC 2011) includes the possibility for review and adaption of the definition to keep up with technical and scientific progress. This review and adaption would take into account definitions agreed upon at Union and international level.

Although this Guidance specifically addresses the use of nanomaterials in medical devices and the generation of nanosized wear-and-tear particles, this Guidance may also be applicable for the evaluation of medical devices containing or generating particles which are not covered by the above definition of nanomaterial (see figure 1). In addition, by analogy, parts of this Guidance may also be useful for the evaluation of nanomaterials when used in medicinal products including tissue engineered medical products."

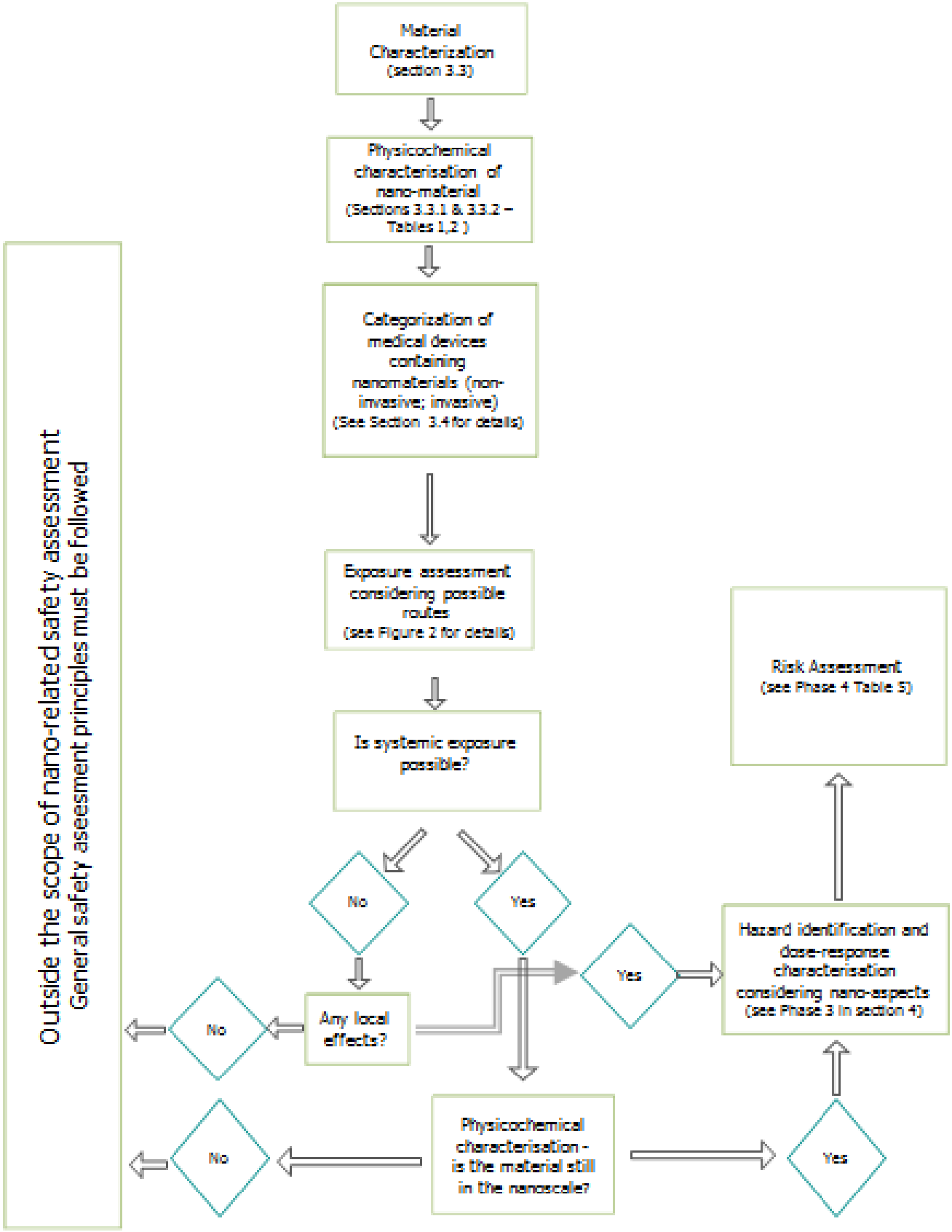


Figure 1: Schematic outline for safety assessment of nanomaterials used in medical devices

## 3.2. Methodology

To prepare this Guidance, SCENIHR reviewed recent scientific evidence to identify the state-of-the-art approaches to perform the safety evaluation and risk assessment of the use of nanomaterials in medical devices.

The SCENIHR has considered evidence from a wide variety of sources, including peer-reviewed scientific and medical literature and published reports of institutional, professional, governmental and non-governmental organisations. As always with the SCENIHR Working Groups, no unpublished works or publicly available opinions that are not scientifically based (SCENIHR 2012) were taken into account.

## 3.3. Characterisation of nanomaterials used in medical devices

Material characterisation of medical devices regarding chemical, physical, morphological and topographical characteristics is defined in ISO 10993-18: 2005, and ISO 10993-19: 2006. ISO/TR 13014:2012 provides information and Guidance on the characterisation of nanomaterials for toxicological screening.

Nanomaterials exhibit unique properties dependent on their size, shape and surface morphology, which are frequently time-dependent. Therefore, nanomaterials require precise characterisation and identification at all stages of design, development, and final production of medical devices containing nanomaterials (SCENIHR 2010, Afssaps 2011, EFSA 2011, SCCS 2012). For risk assessment, this information is essential for the identification of the chemical species under evaluation and for exposure identification. Identification is also necessary to show that the nanomaterials on which the toxicological studies were performed have the same/similar characteristics as those used in the medical device. Justification should be provided if only limited characterisation is possible.

### 3.3.1. Physicochemical characterisation of nanomaterials

The first step in assessing the risks posed by medical devices containing nanomaterials is to chemically identify and characterise the nanomaterials used as starting materials for the production of a medical device, including identifying possible impurities. It is essential to provide evidence that the characterisation data relate to the same nanomaterial that is used in the final product. If the data relate to a different nanomaterial or a different form of the same nanomaterial, justification should be provided that indicates that there is sufficient physicochemical similarity between the nanomaterials to consider the data for risk assessment.

The most important parameters of the nanomaterials intended for use in medical devices are presented in the Table 1, together with suitable characterisation and measurement methods. In addition, ISO/TR 13014:2012 provides information and Guidance on the characterisation of nanomaterials for toxicological screening. Importantly, nanomaterials may change their surface chemistry during processing, for instance by acquiring new or additional surface molecules that may act like coatings. Especially in biological test systems, various proteins are known to adhere to the nanomaterial forming a so-called "protein corona" (Maiorano *et al.* 2010, Lesniak *et al.* 2012). The protein corona is not static as the composition of the proteins adhering to the surface on the nanomaterial changes during contact.

In view of these potential surface changes, it is important that the physicochemical status of a nanomaterial is determined at different stages of testing and/or usage, (EFSA 2011, SCCS 2012), including sterilisation of the invasive medical devices (Lawrence 1998). As the list in the Table 1 is not exhaustive, manufacturers should be ready to provide additional information on other parameters, if necessary, for risk assessment.

**Table 1: Parameters for characterisation and identification of nanomaterials (NM) intended for use in medical devices.**

| Parameter | Description | Methods* |
|---|---|---|
| Chemical composition/ identity | Information on the chemical composition of the NM – including purity, nature of any impurities, coatings or surface moieties, encapsulating materials, processing chemicals, dispersing agents and/or other formulants e.g. stabilizers; information on structural formula(e)/ molecular structure(s) of the constituents of nanomaterial must be provided | MS, AAS, ICP-MS, FTIR, NMR<br><br>UVVis, HPLC, GC/LC-MS, XRD Raman spectroscopy |
| Particle size (Primary/Secondary) | Information on primary particle size, size range and number size distribution (indicating batch to batch variation – if any). The same information would be needed for secondary particles (e.g. agglomerates and aggregates) if present. At least two methods, one being electron microscopy, should be used | FFF, HDC, HPLC, AUC, CLS disc centrifugation, TEM, SEM, STEM, HRTEM, STM, AFM, DLS, DMA, NTA |
| Physical form and morphology | Information on the physical form and crystalline phase/shape. The information should indicate whether the NM is present in a particle-, spherical-, flake-, tube-, rod-, or fibre- shape, the aspect ratio, crystal or amorphous form, and whether it is in free particulate form or in an agglomerated/aggregated state as well as whether the preparation is in the form of a powder, solution, suspension or dispersion. | AFM, TEM, HRTEM, SEM, STEM, STM, NMR, XRD |
| Particle and mass concentration | Information on concentration in terms of particle number and particle mass per volume when in dispersion and per mass when as dry powder. | A wide range of analytical methods, including UV-Vis, HPLC, GC/LC-MS, AAS, ICP-MS |
| Specific surface area | Information on specific surface area of the NM. At the moment this is only applicable for dry powders | BET |
| Surface chemistry | Information on NM surface – including any chemical/ biochemical modifications that could modify the surface reactivity, | LDE, SPM, XPS, MS, RS, FTIR, NMR, AUC (for |

| | | |
|---|---|---|
| | or add a new functionality. | surface composition), GE, SPM, LDE, Nano SIMS, SERS |
| Surface charge | Information on zeta potential of the NM. | PALS (for zeta potential) |
| Redox potential | Information on redox potential, especially for inorganic NMs. Conditions under which redox potential was measured also need to be documented. | Potentiometric methods, X-ray absorption spectroscopy |
| Solubility and partition properties [a] | Information on solubility of the NM in relevant solvents and their partitioning between aqueous and organic phase (e.g. as log $K_{ow}$ if appropriate). | Solubility/ dissolution rate in water and other solvents |
| pH | pH of aqueous suspension. | pH in aqueous media |
| Viscosity | Information on viscosity of liquid dispersions. | OECD 114 |
| Density and pore density | For granular materials, information on density/porosity of unformulated NM and pore density. | DIN ISO 697, EN/ISO 60 |
| Dustiness | Information on dustiness of dry powder products – such as cements and alginates | EN 15051:2006, DIN 33897-2. |
| Chemical reactivity/ catalytic activity[b] | Information on relevant chemical reactivity or catalytic activity of the NM and of any surface coating of the NM. | Kinetic measurements of chemical, biochemical and/or catalysed reactions |
| Photocatalytic activity | Information on photocatalytic activity of relevant materials used (e.g. coatings, dental materials) | TEM, UV, X-ray topography |

* See section 6 Abbreviations and glossary of terms.

a) Dispersion, solution, dissolved: An insoluble nanomaterial introduced to a liquid forms a 'dispersion', where the liquid and the nanomaterial coexist. In a true solution the nanomaterial is dissolved (and thus not present) (see OECD 2012 (ENV/JM/MONO(2012)40).

b) If a nanomaterial has catalytic properties, it may catalyse a redox or other reaction that may perpetuate resulting in a much larger biological response even with small amounts of the catalytically active nanomaterial (see EN 15051:2006, DIN 33897-2). Thus, compared to a conventional biochemical reaction that uses up the substrate, nanomaterial reaction centres may perpetuate catalytic reactions.

### 3.3.2. Methods for characterisation

There are internationally accepted standards for identification and measurements of materials (substances) in their bulk form. Some of these methods can also be used (or adapted) for detection and characterisation of nanomaterials as shown in the Table 1.

In the last decades, various techniques for measuring at the nanoscale were developed, which were mostly based on physical phenomena observed in particle interactions or forces at the nanoscale. Some of the most commonly used techniques are Atomic Force Microscopy (AFM), X-Ray Diffraction, Small-Angle X-ray Scattering (SAXS), dynamic light scattering (DLS), Nanoparticle Tracking Analysis (NTA), and various electron microscopy techniques (TEM, SEM, STEM, HRTEM). These methods for characterisation were

considered in detail in SCENIHR 2010, whereas additional information was provided in the recent ICCR WG report (ICCR 2011), EFSA Guidance (EFSA 2011) and SCCS Guidance (SCCS 2012). The most important conclusion is that size measurement of a particulate material should utilize different techniques depending on whether the nanoparticles occur as a powder, are dispersed in a liquid, are coated or are embedded in a solid material. Not all methods measure the same size, e.g. TEM and AFM measure size without organic coatings, while the size determined by DLS or NTA includes the organic coating in the measurement. Each method has its specific limitations and optimal size range. Nanometrology is defined as the science of measurements at the nanoscale and provides calibration measurements (Proykova *et al.* 2011).

Relevant methods for nanomaterial characterisation may include size separation and extraction (e.g. ultra- centrifugation, FFF, HDC) and chemical analysis / detection by spectroscopic or mass spectrometric techniques, e.g. ICP-MS, UV spectroscopy, AAS, surface area determination (BET) and variants and combinations. Methods for *in situ* imaging of nanomaterials, e.g. magnetic particle imaging (MPI) and positron emission tomography (PET) are currently under development.

Similarly, antibody binding protein and enzyme-based methods are in development for determination of organic or coated-inorganic nanomaterials. Some nanomaterials fall into the class of *metamaterials* for which it is not the composition, but the structure which determines their physicochemical properties (Engheta and Ziokowski. 2006).

Electron microscopy is the most generally applicable method used for nanomaterial characterisation. Size and morphology are readily characterised in the Field Emission Scanning Electron Microscopy (FESEM), FEG-SEM, TEM, STEM and FIB/SEM (see the Table 2). HRTEM allows structural information on particles and atomic clusters to sub-0.2 nm resolution, while EELS and EDX analysis in the STEM allow the chemical analysis of particles down to nanometre diameters. Combining several methods allows for the simultaneous investigation of particle size, shape, structure, composition and surface properties.

**Table 2: Examples of methods for size determination**

| Method | Limitations in range measurements | Phase (liquid, solid, gas) and sensitivity | Particle Distribution |
|---|---|---|---|
| SEM (STEM) | above 50-100 nm | Res. 0.4 nm | no |
| TEM | Few nm | Res. 0.05 nm | yes |
| STM | | Res. 0.01 nm to 0.1 nm | |
| HRTEM | below 0.2 nm | Res. 0.05-0.1 nm | yes |
| AFM | Scanned area is limited | Atomic resolution but sensitivity decreases in time | yes |
| SAXS | 5-25 nm | Res. 0.1 – 0.2 nm | yes |
| DLS & NTA | (1-2000 nm) & (10-15000 nm) | Complex fluids | yes |

More information about various characterisation techniques is provided in the Annex.

Each method for size determination indicated in the Table 2 has specific limitations. Pitfalls in size measuring techniques are indicated in Linsinger *et al.* 2012. An excellent demonstration illustrating the target and the AFM tip change in the course of measurement based on quantum phenomenon can be found in the pictorial available on http://www.loc.ethz.ch/research/grpYamakoshi_EN.

Characterisation and application of nanomaterials in medical devices are not easy tasks. Where nanomaterials are difficult to characterise, a range of methods including characterisation of starting materials, manufacturing process, product performance and toxicokinetic testing may be used as indicated for iron-based nano-sized colloidal medicinal products (EMA, 2013). In addition, the time required to characterise nanomedicines from development through to the *in vivo* application phase is approximately one year. The success rate of Phase 2 human trials (efficacy trials) is 18% in 2008-2010. (Nanotechnology Characterization Laboratory (NCL), USA http://ncl.cancer.gov/). During the "Lessons learned" workshop held in 2011, the NCL presented negative results, "What doesn't work", (Crist *et al.* 2013). Progress in development and characterisation of nanomaterials used in medicine was the focus of the European CLINAM & ETPN Summit, June 23-26, 2013 (Löffler, 2013).

No single method was found that could cover the size range from lower than 1 nm to above 100 nm for all materials. This is one of the reasons that both EFSA and SCCS, in their Guidance, require at least two methods for size determination, one of them being an electron microscopy method (EFSA, 2011; SCCS, 2012). Following this principle, the same requirements apply to the characterisation of nanomaterials used in medical devices.

## 3.4. Uses of nanomaterials in medical devices

The below mentioned applications are examples of current and possible future use of nanomaterials in medical devices, excluding nanotechnologies in medical devices such as nano-electronics and lab-on-a-chip technologies. Etheridge *et al.* (2013) concluded on the use of nanomaterials in medical devices. "The device categories included *in vitro* testing, *in vivo* imaging, *in vivo* device coatings, bone substitutes, dental, medical dressings/textiles, cancer treatment, surgical devices, drug delivery, tissue engineering, and other. *In vitro* testing and *in vivo* imaging were the most prominent categories, followed by *in vivo* device coatings and bone substitutes."
The following are examples of applications of nanomaterials in medical devices that are currently available (Roszek *et al.* 2005; Geertsma *et al.* 2009, ETP 2009, Afssaps 2011, Etheridge *et al.* 2013) and are largely categorised as in ISO 10993-1:2009.

**Examples of devices in current clinical practice:**
**Non-invasive surface contacting medical devices**
These are medical devices, which come into contact only with the intact skin. Examples are operatingantibacterial gowns and textiles to cover patients in the operating theatre, which contain silver nanoparticles.

**Invasive surface contacting medical devices**
These are medical devices, which come into contact with breached or otherwise compromised skin. Examples are wound treatment products (wound dressings) containing nano-sized silver particles and metal oxide particles which are used for improved antibacterial and anti-fungal activity (Vasilev *et al.* 2009, Chaloupka *et al.* 2010).

**Invasive external communicating medical devices**
These are medical devices that come into contact with the blood path, either indirectly or with circulating blood, and devices in contact with tissue/bone/dentin. Examples include:

- Catheters with a nanosilver coating for bladder drainage, haemodialysis and local administering of anaesthesia
- Catheters with nanotopographical morphology imprinted onto the exposed surface
- Polymer based dental composite filler materials and dental cements containing nanoparticles (Ferracane 2011).
- Surgical and dental instruments with nanostructures used to enhance the cutting behaviour and wear resistance of cutting instruments, e.g. scalpels, needles, catheters, burs for cutting bone or teeth
- Instruments with nanostructures used to create non-sticky surfaces to facilitate handling and placement of materials. "Nano-diamond" coatings can be used for this purpose (Dearnaley and Arps 2005).

**Invasive implantable medical devices**
These are medical devices which are intended to be totally introduced into the human body or to replace an epithelial surface or the surface of the eye by surgical intervention, which are intended to remain in place after the procedure. Examples include:

- Carbon nanotubes in bone cements for fixation of implanted prostheses (Van Der Zande *et al.* 2010).
- Bone fillers with hydroxyapatite and tricalcium phosphate nanoparticles which facilitate rapid integration with the bone of the patient.
- Endovascular stents and stent grafts.
- Implants for joint replacement (arthroplasties) and implants for fracture repair.
- Sutures (Ho *et al.* 2013).
- Surface coatings. The surface of implants can be modified with the aid of nanotechnologies to enable them to integrate better in the body (improved biocompatibility) (Mercanzini *et al.* 2010, Thalhammer *et al.* 2010). In addition, coatings can be used for their antibacterial activity.
    - Joint prosthetics (hip, knee) with nanohydroxyapatite coating.
    - Coronary stents with a diamond-like nano composite coating made of ultra-thin polymer.

**Specific types of medical devices**
Injectable medical devices comprise a special category. An example is iron-oxide nanoparticles injected into tumour cells to be heated-up by radiation or an external magnetic field (Vauthier *et al.* 2011; Dutz and Hergt 2013; Torres-Lugo and Rinaldi 2013). Nanoparticles are also being investigated for diagnostic imaging (Skotland *et al.* 2010). However, diagnostic imaging agents are usually classified as Medicinal Products.

For all of the above-mentioned examples, more products from different manufacturers are in development. Examples of applications in development are presented below.

**Examples of applications under development:**

**Non-invasive surface contacting medical devices**
No examples identified yet.

**Invasive surface contacting medical devices**
Silver nanocoatings for various catheters, contact lenses, endotracheal tubes.

**Invasive external communicating medical devices**

- Catheters strengthened with carbon nanotubes for minimally invasive surgery.
- Electrodes with lamina nanocoating through layer-on-layer self-assembly to improve electrode-tissue interface.
- Surface modification of neural micro-electrodes with polymer nanotubes for a low impedance electrode-tissue interface.
- Nanoporous micro-electrodes for a brain-machine interface.

**Invasive implantable medical devices**

- Bone cement/ bone replacement products containing nanosilver as an antimicrobial additive.
- Coronary stents with nanocoatings of aluminium oxide, glycoproteins, hydroxyaptite, platinum or titanium dioxide (Puranik *et al.* 2013).
- Silver nanocoatings for various orthopaedic implants and mesh implants.
- Orthopaedic implants with nanocrystalline metallo-ceramic coatings.
- Modification of the surface roughness of an implant which influences the function of bone-forming and bone-degenerating cells.
- Carrier material ('scaffold') for *tissue engineering products* with a nanoporous structure and surface properties that facilitate the growth of living cells and enable the transport of nutrients, signalling molecules, and waste products. The purpose of these types of products is to replace, repair or regenerate tissues and ultimately, even organs.

**Specific types of medical devices**

- Injectable nanomaterials to be introduced into tumours, which can then be radiated from outside, including:
    - Heat therapy with super paramagnetic iron oxide nanoparticles.
    - Heat ablation with gold nanoparticles.
    - Light therapy.
    - Boron neutron capture therapy.
- Theranostics (therapy combined with diagnostics), i.e. combination of diagnostics and heat therapy with the aid of super paramagnetic iron oxide nanoparticles (because this type of product is still under development, it has not yet been determined if this type of use falls under the legislation on medicines or under the legislation on medicinal devices, however, it is likely that these products will be considered Medicinal Products).

## 3.5. Exposure to nanomaterials from medical devices

Humans may be exposed to nanomaterials from medical devices through various routes. Depending on the relevant exposure route based on the use of a specific medical device, the nanomaterials will encounter various barriers before they are taken up by the body. Patients and users (health care professionals) may be exposed, although the potential of exposure of patients and/or users will differ depending on the particular device and the way it is used. In general, the highest potential for exposure is associated with devices that consist of "free" nanomaterials or that are subject to the release/loosening of nanomaterials present as coatings on the surface of medical devices. In addition, exposure to nanomaterials from medical devices may also result from degradation or wear processes, when nanomaterials are fixed on the surface (e.g. as coating on implants) or are embedded within the material of the medical device.

A great variety of nanomaterials is used in nanomedicine, including structures based on lipids, proteins, DNA/RNA or other naturally occurring materials and substances, and

those based on polymers, both degradable and non-degradable. Other groups of particles used in medical devices that may contain nanomaterials are pigments and fillers. The various known forms of carbon-like carbon nanotubes (CNT), diamond, carbon black, carbonfibres, and carbonwires are also frequently used. Furthermore, many different sorts of metals and metal oxides are used, as well as silica, quantum dots and a number of specific types that do not easily fit in a larger category.

In general, therapeutic devices, sensors/diagnostics for *in vivo* use, regenerative medicine, and implants result in high exposure potential for patients. For professional users, exposure potential is low. When the nanomaterial is used in an unbound (free) state, it can potentially spread through the body.

In the ISO 10993 series, the following standards focus on the characterisation of medical devices and their degradation products. Although nanomaterials are not addressed in these standards, they provide information on the general characterisation of the various components used in medical devices.

ISO 10993-9:2009. Biological evaluation of medical devices – Part 9: Framework for identification and quantification of potential degradation products.

ISO 10993-13:2010. Biological evaluation of medical devices – Part 13: Identification and quantification of degradation products from polymeric medical devices.

ISO 10993-14:2001. Biological evaluation of medical devices – Part 14: Identification and quantification of degradation products from ceramics.

ISO 10993-15:2000. Biological evaluation of medical devices – Part 15: Identification and quantification of degradation products from metals and alloys.

ISO 10993-18:2005. Biological evaluation of medical devices – Part 18: Chemical characterization of materials.

ISO 10993-19:2006. Biological evaluation of medical devices - Part 19: Physicochemical, morphological and topographical characterisation of materials.

### 3.5.1. Release of nanomaterials from medical devices

In general, the highest potential for release of nanomaterials from medical devices is associated with devices

- in which the nanomaterial is intended to be released,
- that are composed of free nanomaterials, e.g. ironoxide nanoparticles for heat therapy and/or
- containing free nanomaterials, e.g. nanosilver as used in wound dressing, nanomaterials present in bone fillers.

Another possibility for release of nanomaterials from medical devices is associated with release/loosening of nanomaterials present as coatings on medical devices as well as chemical breakdown or wear-and-tear processes due to (bio)degradation of medical devices containing nanomaterials. Additionally, in some applications, medical devices must be grinded, polished or shaped during application, e.g. dental fillings, which may be a source for the release of nanomaterials.

Notably, there are medical devices containing free nanoparticles during a very short exposure time. For most of the exposure time, these devices contain firmly bound nanomaterials, e.g. dental fillings or bone cements that are cured during application to transform them from a paste to a solid form. When there is a strong bonding on the

medical device surface or when the nanomaterials are firmly incorporated in the matrix of a (bio)material, no nanoparticles, or a negligible amount, are released.

Chemical breakdown of degradable materials results in nanomaterial release when the nanomaterial is present as coating or embedded in the degradable matrix. Another scenario for nanoparticles release is in the case of a composite nanomaterial, which is exposed to both mechanical and chemical wear-and-tear.

Nanoscale particles may be generated as a consequence of the degradation of medical devices not containing nanomaterials. Bulk materials, either solid or porous, can degrade due to hydrolysis or catalysis. Eventually, the degradation may lead to the production of particles, which may be nano-sized. For materials that intentionally or unintentionally degrade upon tissue contact, particles will ultimately be formed as a result of mechanical collapse, which may result in the generation of nanoparticles from either the bulk material or nano-sized components.

Nanoparticles may be generated through abrasive wear or grinding of a material (Frogget *et al.* 2014). Several scenarios could be identified for nanoparticle release including machining, weathering, washing, contact and incineration (Frogget *et al.* 2014). Identified debris contained particles from matrix alone, matrix particles with the nanomaterial embedded, the nanomaterials themselves or dissolved ionic forms of the added nanomaterial. An example of this are resin-based composites used in restorative and aesthetic dentistry. This type of composite with nano-sized fillers of various sizes and shapes has been increasingly used in recent years due to superior aesthetic and mechanical properties. At the same time, particles in the nano size range were detected in debris after grinding or polishing dental composites on a laboratory surface as well as in the aerosol after polishing of nano-composite restorations in the front teeth (Van Landuyt *et al.*2012, 2014; Bogdan *et al.* 2014). The released nanosized materials do not necessarily contain the original nanomaterials present in the medical device. They may be covered with material from the matrix (Bogdan *et al.* 2014). In addition, when incorporated in a matrix, nanomaterial toxicity may be different from the toxicity of the original pristine nanomaterial (Smulders *et al.* 2014). There are only a limited number of occupational exposure limits for nanoparticles (e.g. nanoscale $TiO_2$, carbon nanotubes and fibers, NIOSH 2011, 2013), making it impossible to speculate on relative health associated risks from nanoparticles released when grinding or polishing dental composites. There is also a lack of information necessary in order to establish such limits.

Joint articulations using metal-on-metal as well as metal-on-polyethylene sliding surfaces produce wear particles being the most frequent reason for revision surgery (SCENIHR, 2014). For metal debris, the particle size is less than 1 µm for most (>90 %) of the particles, while for polyethylene, most particles were above 1 µm (Lee *et al.* 1992). Notably, the distribution of particles in several reports show the largest number of particles in the smallest analysed category indicating that nano-sized particles are most likely to be present. A generic all-encompassing term "adverse reactions to metal debris" (ARMD) was introduced that summarises the histopathology associated with metal-on-metal hip prostheses including aseptic lymphocytic vasculitis-associated lesions, lymphoid neogenesis, granulomatous inflammation and metallosis (Natu *et al.* 2012).

In all circumstances in which there is a possibility for the generation and release of (nano)particles, a careful characterisation of the particles is necessary according to the methods described in section 3.3.

### 3.5.2. Exposure routes to nanomaterials released from medical devices

For patients, the following exposure routes may be applicable:

- inhalation exposure, e.g. related to intubation, dental procedures;
- dermal exposure;
- mucosal exposure (via various mucosal tissues, e.g. in the mouth, vagina/penis);
- oral exposure;
- parenteral exposure (introduced into the body by a means other than through the gastro-intestinal tract, e.g., by injection into the bloodstream (intravenous) or a muscle (intramuscular), surgical procedures using medical devices or from implanting devices in any tissue;
- ocular exposure.

#### 3.5.2.1 Non-invasive medical devices

These are devices in contact with intact skin. Released nanosized components have a low potential to penetrate through the skin (Labouta and Schneider 2013) (see section 3.6.3).

Note: the use of contact lenses on the surface of the eye is considered under the medical devices regulations as an invasive medical device.

#### 3.5.2.2 Invasive medical devices

All classes of invasive devices may potentially generate nanoparticles. For invasive devices, the released nanoparticles have a direct port of entry into the body depending on the localisation of the device used.

Products consisting of free nanomaterials lead to high potential for systemic exposure, i.e. to the entire body, regardless of the administration route (oral, dermal, parenteral or intravenous). Whether a high systemic exposure occurs depends on the actual use of the medical device and the route of exposure, i.e. the location where the medical device is used.

Nanomaterials in products used in surgery are generally embedded inside or coated on larger products. The duration of contact with the patient is relatively short. Local exposure to the bound nanomaterials at the site of treatment will, therefore, be high in all cases, whereas systemic exposure potential to free nanomaterials is likely to be very low. For implants, nanomaterials are usually embedded or fixed on the surface. Duration of contact is long-term. Local exposure to the fixed nanomaterials at the site of treatment will therefore be high in all cases, whereas systemic exposure potential to free nanomaterials may be considered low, provided there is only slow generation of wear particles. Exposure may also occur during the treatment procedures with dental composite materials cured *in situ*, bone and tissue fillers containing nanomaterials. Especially for dental fillings, exposure may also occur during polishing. (Van Landuyt *et al.* 2014, Bogdan *et al.* 2014).

### 3.5.3. Exposure of users to nanomaterials released from medical devices

For professional users, e.g. dentists and dental technicians, the potential exposure is highest when free nanomaterials are present in the medical device, e.g. in certain dental composite materials and bone fillers. Exposure may especially occur during polishing of dental fillings (Van Landuyt *et al.*2014, Bogdan *et al.* 2014).

For these professional users, the following exposure routes may be applicable:

- inhalation exposure (e.g. related to dental procedures);
- dermal exposure;
- mucosal exposure (via various mucosal tissues, e.g. in the mouth);
- oral exposure;
- ocular exposure.

### 3.5.4. Estimation of exposure for risk assessment

Based on the potential exposure to nanomaterials in medical devices, an estimation can be made of the exposure using the exposure times and the exposure categories used in the risk assessment and risk management of medical devices indicated in ISO 10993-1:2009 and ISO 14971:2007 (see Table 3).

Three exposure categories of devices are considered based on the application site of a medical device:

- surface contacting device;
- external communicating device;
- implant device.

The types of tissue contact considered in the risk assessment includes these categories:

- skin;
- mucosal membrane;
- breached or compromised surface;
- blood;
- tissue;
- bone;
- dentin.

The contact time must be considered:

- limited contact (≤24 hours);
- prolonged contact (> 24 hours to 30 days);
- permanent >30 days.

In addition to the potential (bio)degradable property of a material, the "quality" of the material used to manufacture a medical device should be considered in terms of possible resistance against wear-and-tear.

Importantly, measuring the release of nanomaterials from a medical device may pose analytical challenges. Currently, a robust methodology especially for the measurements of low level release of nanomaterials is lacking. For metal and metal oxide nanomaterials, elemental analysis may be used as a surrogate for nanoparticle and/or ion release.

**Table 3: An estimation of potential external and internal exposure as starting point for a risk evaluation for medical devices containing nanomaterials**

| | | | Type of application of nanomaterials External exposure/internal exposure | | | | |
|---|---|---|---|---|---|---|---|
| | | | **Free** | **Fixed (coating)** | **Fixed (coating)** | **Embedded** | **Embedded** |
| **Type of device** | **Type of contact** | **Duration of contact** | | **Weak (physisorb)** | **Strong (chemisorb)** | **In degradable materials*** | **In non-degradable materials** |
| Surface device | Intact skin | ≤ 24 h | H/N | M/N | M/N | L/N | N/N |
| | | >24 h to 30 d | H/N | M/N | M/N | M/N | N/N |
| | | >30 d | H/N | M/N | M/N | H/N | N/N |
| | Intact mucosal membrane | ≤ 24 h | H/L | M/L | M/N | L/L | N/N |
| | | >24 h to 30 d | H/M | M/M | M/L | M/M | N/N |
| | | >30 d | H/M | M/M | M/L | H/M | N/N |
| | Breached or compromised surface | ≤ 24 h | H/H | M/M | M/L | L/M | N/N |
| | | 24 h to 30 d | H/H | M/M | M/L | M/M | N/N |
| | | 30 d | H/H | M/M | M/L | H/M | N/N |
| External Communicating device | Blood path, indirect ** | ≤ 24 h | na | M/M | M/L | L/L | N/N |
| | | >24 h to 30 d | na | M/M | M/L | M/M | N/N |
| | | >30 d | na | M/M | M/L | H/M | N/N |
| | Tissue/bone/ dentin | ≤ 24 h | H/H | M/M | M/L | L/L | N/N |
| | | >24 h to 30 d | H/H | M/M | M/L | M/M | N/N |
| | | >30 d | H/H | M/M | M/L | H/H | N/N |
| | Circulating blood*** | ≤ 24 h | na | H/H | H/H | L/L | N/N |
| | | >24 h to 30 d | na | H/H | H/H | M/M | N/N |
| | | >30 d | na | H/H | H/H | H/H | N/N |
| Implant device | Tissue/bone | ≤ 24 h | H/H | H/H | H/L | L/L | N/N |
| | | >24 h to 30 d | H/H | H/H | H/L | M/M | N/N |
| | | >30 d | H/H | H/H | H/L | H/H | N/N |
| | Blood | ≤ 24 h | H/H | H/H | H/L | L/L | N/N |
| | | >24 h to 30 d | H/H | H/H | H/L | M/M | N/N |
| | | >30 d | H/H | H/H | H/L | H/H | N/N |

H=high, M=medium, L=low, N=negligible, na= not applicable

H/L means high potential contact and/or external exposure to the nanomaterial / low potential for internal systemic exposure of all organ systems

* the exposure will depend on the degradation time of the medical device

** contacting the blood path at one point. Examples of these types of devices are solution administration sets, transfer sets and blood administration sets (ISO 10993-4:2002)

*** Examples of these types of devices are: intravascular catheters, extracorporeal oxygenating tubing and dialysers (ISO 10993-4:2002).

## 3.6. Toxicokinetics

### 3.6.1. Introduction

Toxicokinetic testing gives information on the fate and behaviour of the substances in evaluation and provides insight into potential target organs and organ burden that may ultimately result in toxicity.

The toxicokinetic properties of nanomaterials, like other substances, can be described by four processes: absorption, distribution, metabolism and excretion (ADME). The study is essential for the safety evaluation of engineered nanomaterials. The nature of nanomaterials may alter the toxicokinetics and tissue distribution when compared to non-nanoforms (EFSA 2011, SCCS 2012). For subgroups of certain solid nanomaterials, it is doubtful whether metabolism (M) really occurs. Although for some nanomaterials, metabolism was reported (Landsiedel *et al.* 2012). Tissue distribution, accumulation and elimination from tissues are considered more relevant than blood plasma levels. It is particularly important to evaluate nanomaterial presence in typical distribution organs (and thus potential targets for toxicity) that have an increased capacity for uptake of particles, e.g. liver, spleen, and lungs (EFSA 2011). In addition, the kidney is an important organ because of possible excretion of the nanomaterials.

The route of entry is important because it may affect the kinetics of nanomaterials/nanoparticles, e.g. Au nanoparticles (1.4 nm) showed a higher uptake in the kidney compared to the liver after intratracheal administration. In contrast, the liver was the predominant target organ after intravenous administration, suggesting alteration of the nanoparticles during passage of the air/blood barrier in the lung (Oberdörster 2010, Semmler-Behnke *et al.* 2008). Depending on the site of application, further kinetics of a released nanomaterial may be affected by adherence of molecules to the surface of a nanomaterial. In this respect, the formation of a serum protein "corona" that is suggested to enhance recognition and uptake by cells of the mononuclear phagocyte system (MPS) is well known (Lynch *et al.*2009, Lynch and Dawson 2008, Nel *et al.* 2009). The cells involved are primarily monocytes and macrophages present in various organs of the immune system like spleen, lymph nodes and bone marrow. Additionally, tissue macrophages like Langerhans cells in the skin, Kupffer cells in the liver and alveolar macrophages in the lung are part of the MPS. In general, the clearance of the nanoparticles from the blood is mainly via the liver and spleen (De Jong *et al.* 2008, Demoy *et al.* 1997, Gibaud *et al.* 1996, Lenaerts *et al.* 1984, Sadauskas *et al.* 2007, Lipka *et al.* 2010, Lankveld *et al.* 2010, 2011).

Locally released nanoparticles in tissues may migrate or be transported into the systemic circulation. The primary transportation system is the lymphatic, which allows for transportation of the particles, but also of particles that have been phagocytised by tissue macrophages and/or inflammatory cells. Regional lymph nodes are the primary recipient for these particles. However, depending on the primary localisation, and upon entry into the blood circulation, the nanoparticles may end up in other organs such as the spleen and the liver. Nanomaterials released from a medical device can translocate from their site of origin into the body.

The route of exposure depends on the medical device used. Potentially, all routes of exposure are possible. Independent of the route of exposure for medical devices, the absorption and bioavailability of potentially released nanomaterials from a medical device, or the generation of nanoparticles via wear-and-tear (Polyzois *et al.* 2012), are the starting points for evaluation of the toxicokinetics of nanomaterials.

### 3.6.2. Methods to evaluate toxicokinetics of nanomaterials

The design and performance of toxicokinetic studies for chemicals, degradation products and leachables from medical devices is described in ISO 10993-16:2010, although degradation products that may be considered nanoparticles are not mentioned. The OECD 417 test guideline describes the toxicokinetic studies performed for chemical substances and explicitly states that it is not intended for the toxicokinetic testing of nanomaterials. Analogously, both the *in vivo* and *in vitro* OECD Guidelines 427 and 428 for dermal penetration, respectively, were developed for chemicals and not proven valid for nanoparticles. Therefore, the use of such methodologies should be evaluated on a case-by-case basis.

For a dissolved chemical, tissue uptake and release is generally dependent on the blood concentration (when excluding specific active transport, the first-pass effect in the liver and highly bioaccumulating chemicals in the adipose tissue) and an equilibrium between blood and organ concentration is generally obtained. Nanoparticle uptake in organs occurs rapidly and repeated administration results in an increase of nanomaterials, predominantly in the liver and spleen after intravenous administration (Lankveld *et al.* 2010). The biodistribution of nanoparticles is influenced by a number of factors including size, surface charge, and surface composition like protein binding and coating (De Jong *et al.* 2008, Lankveld *et al.* 2011, Landsiedel *et al.* 2012). There is no equilibrium concentration between tissue and blood. Uptake in organs can occur independent of the blood concentration, i.e. even with a low blood concentration and high organ concentration, organ uptake can occur. These results in the persistence of nanomaterials in organs for long periods: silver could still be detected in various organs at day 17 after intravenous administration of silver nanoparticles in rats (Fabian *et al.* 2008; Pauluhn 2009; Lankveld *et al.* 2010). Titanium nanoparticles were still detectable up to 90 days after a single and repeated intravenous administration (Nanogenotox 2013, Geraets *et al.* 2014). To identify tissue distribution and the potential for tissue accumulation and persistence of a nanomaterial, it is necessary to design single and repeated kinetic studies with a representative follow-up period of time. This information is useful to adequately extrapolate the half-life time (Lankveld *et al.* 2010, Geraets *et al.* 2014). In OECD 417 on toxicokinetic testing, the follow up period is typically up to 7 days, which may be too short a period for nanomaterials in view of their potential persistence in organs.

Release/elimination from an organ seems to be associated with a possible dissolution or degradation of the nanomaterials. For some nanomaterials, (quantum dots, Polystyrene nanoparticles, MWCNT) excretion was demonstrated by the kidney and or liver (Landsiedel *et al.* 2012). Potential persistence occurs especially for non-degradable solid nanomaterials.

To conduct (toxico)kinetic studies with nanomaterials, adapting the known test usually used for chemicals or bulk forms, the critical point is the availability of a measurement system for detection of the nanomaterials. However, detection of nanoparticles in tissues/organs is complex. Electron microscopy is neither applicable for quantitative measurements nor for all nanomaterials. To date, most studies on toxicokinetics of nanomaterials have used elemental analysis of the components of the nanomaterials e.g. Zn for ZnO, Ti for TiO2, Ag for Ag nanoparticles. Analysis could be performed by using inductively coupled plasma mass spectroscopy (ICP-MS) or atomic absorption mass spectroscopy (AA-MS). Although this provides a good indication of the possible tissue distribution, the limitation is that the nanoparticles themselves are not detected or measured. In combination with separation techniques like field flow fractionation (FFF) it is possible to evaluate the presence of particles using so called single particle ICP-MS (Van Der Zande *et al.* 2012).

Specific labelling of nanomaterials to follow their fate *in vivo* can be done by using radioactive isotopes as radiolabels or fluorescent dyes. A disadvantage of these forms of

labelling is that the label can detach from the nanomaterial (Geiser and Kreyling 2010). The measurement or imaging will then identify the label, but not the distribution of the nanoparticle. Alternatively radioactive isotopes may be used that are isotopes of a metal being part of the nanomaterial (e.g. gold or silver). With this approach, there is some certainty that the nanoparticles themselves are detected, although for silver nanoparticles there is still uncertainty regarding the release of silver ions. In addition, natural stable isotopes like $^{68}$Zn may be used to demonstrate uptake from the application site (Gulson *et al.* 2010).

There is uncertainty whether the nanomaterial or the released ions are detected, especially when a nanomaterial can release ions (e.g. silver or zinc oxide). After skin application of sunscreens containing $^{68}$Zn isotope enriched ZnO nanoparticles, the $^{68}$Zn could be detected in the blood of humans and in internal organs, including the liver in mice but skin penetration of the ZnO nanoparticles themselves could not be established (Gulson *et al.* 2010, Osmond-McLeod *et al.* 2013).

Surface treatments can have a tremendous effect on the toxicokinetics of nanomaterials. The PEGylation (coating a nanomaterial with poly-ethyleneglycol) decreased the blood clearance of intravenously administered gold nanorods (Niidome *et al.* 2006, Lankveld *et al.* 2011). Additionally, specific targeting to organs may be achieved by the coating of nanomaterials.

### 3.6.3. Toxicokinetics of nanomaterials present in non invasive medical devices

**Uptake after dermal exposure**

Assessment of dermal penetration can be performed using *in vitro* systems for which the skin of many mammalian species, including humans, may be used as indicated in OECD 428. *In vivo* skin absorption studies are described in OECD 427. However, none of these tests were designed for nanoparticles. Nanoparticle quantitation remains a problem in these studies. Dermal penetration of nanoparticles is generally considered to be low or absent (Butz *et al.* 2007, Monteiro-Riviere and Riviere 2009b, Sadrieh *et al.* 2010, Monteiro-Riviere and Larese Filon 2012). In general, nanoparticle penetration of the skin is limited to the first cell layers of the stratum corneum (Butz *et al.* 2007). However, for some nanomaterials, there seemed to be limited uptake. For example, when ZnO nanomaterial was applied on the skin in a sunscreen formulation, the presence of Zn in the blood originating from the ZnO in the sunscreen was observed (Gulson *et al.* 2010).
Silver (Ag) nanoparticles are widely used as antimicrobial agents, for example in wound dressings (Rai *et al.* 2009, 2014). In an *in vitro* system using human skin exposed to Ag nanoparticles, a low translocation into the receptor fluid was found which was increased fivefold in damaged skin (Larese Filon *et al.* 2009). However, it could not be clearly demonstrated that nanoparticles were translocated, because the presence of elemental Ag was determined with electrothermal atomic absorption spectroscopy (ETAAS) which cannot discriminate between silver ions and silver particles. Treatment of burn patients with wound dressings containing nanocrystalline silver increased blood silver serum levels, although these levels were considered to be non-toxic to the patients (Vlachou *et al.* 2007).

For skin penetration and absorption, the quality of the skin in terms of skin damage, like abrasions and UVB damage (sunburns), mechanical stressors (skin flexing), and the effects of solvents and vehicles used may affect the skin penetration (Monteiro-Riviere and Larese Filon 2012).

### 3.6.4. Invasive medical devices

**Uptake after ocular exposure (via the eye)**
Nanomaterials could be used in contact lenses. However, there are no data available regarding the release and kinetics of such nanomaterials. In general, for the eye, the use of various nanomaterials is aimed at enhancing the uptake and targeting drugs into the eye. In a recent review, the therapeutic efficacy of drugs in ocular diseases was enhanced by the use of nanoparticles such as liposomes, micro/nanospheres, microemulsions, and dendrimers (Honda *et al.* 2013). The nano-property of these products may disappear after application. For chitin containing nanogels, penetration into the deeper sections of the porcine cornea was observed without signs of destruction or inflammation to corneal cells (Mohammed *et al.* 2013).

**Uptake after inhalation exposure (e.g. related to dental procedures)**
The inflammatory effect of particles on the lung is quite well known as a result of many inhalation studies with particles and nanomaterials. After exposure via the inhalation route by either inhalation or instillation, a small but significant fraction of the dose of nanoparticles may be detected systemically, although the majority of the nanoparticles remained in the lung (Kreyling *et al.* 2002, Semmler-Behnke *et al.* 2008, Sung *et al.*2011, Abid *et al.* 2013). The elimination half-time from the lung for both fine and ultrafine (nano)particles in rats was approximately 65 days (Pauluhn 2009, 2011). Due to the mucociliary cascade that removes inhaled particles from the lung, a portion of the inhaled/instilled nanomaterials ended up in the gastro-intestinal tract (GI-tract) and was excreted in the faeces (Abid *et al.* 2013). In addition, certain inhaled nanomaterials may migrate into the brain via the olfactory nerve (Oberdörster *et al.* 2004, Balasubramanian *et al.* 2013). The primary particle size of the nanoparticles was important as smaller (7 nm versus 20 nm) nanoparticles had a higher uptake from the lung (Balasubramanian *et al.* 2013). In this study, macrophage-mediated mucociliary escalation, followed by faecal excretion, was the major pathway of clearing the inhaled nanoparticles from the lungs.

**Uptake after oral exposure**
Uptake from the GI-tract was demonstrated for several nanomaterials (Jani *et al.* 1990, 1994, Wang *et al.* 2007, Kim *et al.* 2008, Park *et al.* 2010 a, b), but a lack of uptake of nanoparticles was also observed (Yang *et al.* 2012). In general, smaller particles were found to have a higher uptake (Jani *et al.* 1990, Park *et al.* 2010a). However, large titanium particles with a size of 500nm were also absorbed via the GI-tract (Jani *et al.* 1994).

**Uptake after transdermal exposure (implants)**
When present on or in medical devices that penetrate the skin, the local release of coatings consisting of nanomaterials may be possible. In practice, transdermal and other implants will most likely generate only a minor amount of locally released nanoparticles, an exception being wear-and-tear occurring after arthroplasties. Thus, the subcutaneous administration of nanomaterials may be an alternative for studying particle distribution. Following subcutaneous injection, the largest particle agglomerates were found mainly in the draining inguinal lymph node, and to a lesser extent, the liver, spleen and lung (Umbreit *et al.* 2011).

### 3.6.5. Conclusions on toxicokinetics of nanomaterials

The performance of toxicokinetic studies to evaluate tissue distribution and kinetics of nanomaterials are indicated when there is the possibility for the release of free

(nano)particles from a medical device. Although methods used for chemicals in bulk form can be adapted, specific attention should be given to the detection method. Blood clearance generally appears quite quickly, thus blood levels are less important than the ultimate tissue and organ levels. In addition, consideration should be given to the potential for tissue accumulation and persistence of a nanomaterial (e.g. dissolution/degradation of the nanomaterial), for which repeated exposure and prolonged follow-up time may be necessary. In general, after systemic availability, the clearance of the nanoparticles from the blood is into the organs of the MPS mainly liver and spleen. So, when release of nanomaterials is likely, possibilities for translocation and uptake/persistence in organs rich in phagocytic cells (e.g. liver, spleen, bone marrow) should also be considered.

## 3.7. Toxicological evaluation

### 3.7.1. Introduction

The toxicity testing strategy of an individual medical device containing nanomaterials is determined by its potential of external and internal exposure. Therefore, hazard evaluation has to be performed on a case-by-case basis, through a series of studies including literature review, *in silico*, *in vitro* and *in vivo* studies. For medical devices selection of any *in vitro* or *in vivo* tests should be based on end-use applications. All tests should be conducted according to recognised current/valid best laboratory/quality practices, for example Good Laboratory Practice (GLP) or ISO/IEC 17025, where applicable, and the data should be evaluated by competent informed professionals (ISO 10993-1). The needed toxicity studies should be performed in accordance with the International Standards ISO 10993 series (ISO 10993 – 1, 3-6, 10-12, 17, 19). *In vivo* studies should be performed using an administration route which is relevant to the route of human exposure to the medical device and/or nanomaterials. However, it should be emphasised that none of currently available test methods, both *in vitro* and *in vivo*, have been validated specifically for nanomaterials. Materials in nanoform pose many challenges when tested; unlike solubilised chemicals, nanomaterials generally exist as a suspension/dispersion of insoluble or partially-soluble nanoparticles and/or larger agglomerates and aggregates, which may affect the test system.

The degree of nanomaterial toxicity depends upon the particle size and additional specific characteristics, most of them listed in Table 1. Therefore, it is essential that tests are conducted using the same nanomaterial with the same chemical composition, size and size distribution, surface properties and purity/impurity profile as the substance present in the medical device, and should, therefore, be characterised before testing. Thus, the information on the nature and stability of the test substance under experimental conditions is of prime importance for the interpretation of any test results. If a comparable/similar (nano)material is used, this should be justified and documented.

Because nanomaterials may acquire new 'biological identities', i.e. new properties via the adsorption of biomolecules (the bio-corona) onto their surface, it is essential during toxicological studies to assess the interaction between these and how these may interact with the physiological response of the organism, (Fadeel *et al.*2013). In addition, some unexpected toxicities may be induced by nanomaterials as for example was indicated by the induction of autophagocytosis markers by silver nanowires (Verma *et al.*2012). However, for granular biodurable particles without known specific toxicity (GBP), there was no valid indication that GBP nanomaterials possess novel toxicological hazard properties (as reviewed by Moreno-Horn and Gebel 2014).

There are ongoing developments in *in vitro* methods, but currently there are no validated *in vitro* methods for hazard assessment of nanomaterials (Park *et al.*2009, Cockburn *et al.*2012, Doak *et al.*2012, Nel *et al.*2013a). However, *in vitro* tests may be useful for screening purposes and to elucidate possible mode of action (Basketter *et al.*2013, Nel *et al.*2013b), but their use should be evaluated on a case-by-case basis. For the induction of fibrosis and epitheloid cells by high aspect ratio, HAR, nanomaterials, e.g. some CNTs models were also developed to study the mechanisms of nanomaterial cell interaction (Sanchez *et al.*2011, Vietti *et al.*2013). A catalogue of all currently validated *in vitro* methods is published on:
http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/validation-regulatory-acceptance/,

Whilst *in silico* modelling approaches are advancing for conventional chemicals, a relationship between the various physicochemical properties and toxicological effects of nanomaterials has not yet been investigated and established to allow development of reliable models for nanomaterials. As a result, only a few rudimentary *in silico* models are currently available for nanomaterials (Toropov and Leszczynski 2007, Toropov *et al.*2007, Puzyn *et al.*2009, 2011, Sayes and Ivanov, 2010; Burello and Worth, 2011, Wang *et al.*2014). In a recent review, some limitations were identified for the use of QSAR techniques in nanotoxicology (Winkler *et al.*2013). They stated the following on the use of *in silico* techniques. "Three of the major roadblocks to applying QSAR methods to modelling biological properties of nanoparticles are insufficient experimental data on the composition of the bio-corona on nanoparticle surfaces, the lack of *in vitro* data predictive of *in vivo* effects of nanomaterials, and the paucity of 'nanoparticle-specific' descriptors." (Winkler *et al.*2013). Although, there has been progress in the development of *in silico* models, they are unlikely to be useful in the foreseeable future for the assessment of relevant toxicological endpoints that are needed for risk assessment. In addition, "omics" techniques look promising as an alternative approach for safety testing of medical devices and/or nanomaterials in the future.

### 3.7.2. Potential pitfalls in toxicity testing of nanomaterials

Following the ISO 10993-1:2009 standard regarding the evaluation and testing of medical devices within a risk management process, the toxicity testing strategy for each device should be considered case-by-case based on the type of medical device, type of contact and duration of exposure. Most of the toxicity assays as described in the various parts of the EN-ISO 10993 series are developed specifically for medical devices, and are based on the OECD Guidelines for the testing of chemicals.

Testing of insoluble or partially-soluble nanoparticles using *in vivo* or *in vitro* methods must also take into account that they will be present in a dosing or test medium as a nano-dispersion rather than in solution. Therefore, any toxicity testing using *in vivo and in vitro* methods should pay special attention to the agglomeration/aggregation behaviour, and the insoluble/ partially-soluble nature of nanomaterials (SCENIHR, 2009; Kreyling *et al.*2010, EFSA 2011, SCCS 2012). Possibilities for disagglomeration and re-aggregation of nanomaterials should also be considered. During toxicological evaluations, some properties of nanomaterials may change due to interaction with the surrounding media.

Special care is therefore needed in regard to the applied doses, which can be affected by the above mentioned phenomena. In addition, the concentration of a nanomaterial may decrease during a test due to sedimentation, binding with other moieties in the test medium, or adhesion to glass/plastic ware. It is therefore important to ascertain the stability and uniformity of the nanomaterial in a test medium to ensure that the applied concentration/dose is maintained for the intended period during the test. In certain assays, high concentrations *in vitro* or excessive doses *in vivo* can lead to a false interpretation of results, e.g. overload dosing in inhalation studies (Valberg *et al.*2009).

Importantly, physicochemical properties may be affected if vehicle, test or cell culture medium results in adsorption of biomolecules on the surface of the nanomaterial. These acquired coatings may influence general toxicity. Therefore, a proper characterisation under dosing/test conditions is needed. In addition, it is important to ascertain the stability and uniformity of the nanomaterial in a test medium to ensure that the applied concentration/dose of nanomaterial is as assumed (Allouni *et al.*2009).

When testing nanomaterials, the presence of endotoxin in the nanomaterial dispersion as indicator for possible bacterial contamination should be excluded. Endotoxin may interfere with the test system and may lead to false negative or positive results depending on the test system.

Importantly, there may be an interaction between test reagents and the nanomaterials especially in colorimetric assays (such as sulforhodamine B dye, or MTT used in the viability assays) (Worle-Knirsch *et al.*2006, Monteiro-Riviere *et al.*2009a, Lupu and Popescu 2013). Moreover, some nanomaterials may themselves disperse/ absorb light and therefore interfere with the measurements in colorimetric assays. These aspects need to be considered when using colorimetric methods. Produced proteins/biological mediators (e.g. cytokines) may also bind/adsorb on nanomaterial surfaces and may lead to low responses or even false negative results (Worle-Knirsch *et al.*2006, Monteiro-Riviere *et al.*2009a, Val *et al.* 2009; Wilhelmi *et al.*2012). Some of these problems might be overcome by either adding appropriate controls or modifying existing protocols. For instance, nanomaterials have been shown to interfere with the optical density readings for tetrazolium-based assays such as MTS and MTT; however, removal of nanomaterials via centrifugation before reading the assay can reduce the variations in data generated for the same nanomaterials (Xia *et al.*2013, Ong *et al.*2014).

Some metals (silver) or metal oxides (ZnO) undergo (slow) dissolution in media, therefore, part (or all) of the activity measured might be due to the dissolved ions. For those types of nanomaterials, determination of the solubilised fraction before and during testing might be warranted. In some assays, adding a suitable control in the ionic form should be considered.

The harmfulness of nanomaterials may arise from their size-related ability to readily enter biological systems and modify the structure of proteins through formation of new protein complexes or enhanced protein degradation (Lovric *et al.*2005, Aggarwal *et al.*2009, Mailander and Landfester 2009).
Nano-sized particles are likely to be phagocytised by inflammatory cells, especially macrophages and polymorphonuclear neutrophils. Whether the particles are in aggregated or non-aggregated suspension is critical and notably these aggregates and agglomerates may be larger than the nano-size range (i.e. 100 nm). Although aggregates/agglomerates smaller than 100 nm may also be present. Affinity for and subsequent adsorption of proteins and peptides may change the biological significance and enhance triggering of inflammatory humoral and cellular processes. Endocytosis of spherical NPs is easier and faster compared to rod-shaped or fiber-like nanomaterials (Champion and Mitragotri 2006). Rod-shaped or needle-like NPs may have a larger contact area with the cell membrane receptors than spherical NPs when the longitudinal axis of the rods interacts with the receptors. Hence, the ends with high curvature at the half-cup stage of endocytosis are very likely to cause a higher membrane surface energy, resulting in a large distorting force that exceeds the maximum force provided by the actin polymerisation. This effect stalls the growing ends of the phagocytic cup and results in impaired phagocytosis and the macrophage spreading onto the material rather than internalizing it (Lu *et al.*2010).

The metrics used for toxicity assessments are normally measured and expressed in weight or volume units (such as mg/Kg, or mg/L) for conventional chemicals. However, such dose metric may not be appropriate for nanomaterials, because of the large surface

areas per particle mass or volume. For nanomaterials, surface area or number of particles might give a better description of a possible dose-response effect relationship. Nanoparticle shape can modify activity as well. Until suitable parameters are identified, it is important that different dose-describing metrics, such as weight/volume concentration, particle number concentration, surface area etc. are available to have sufficient information to converse doses based on mass into other parameters (Donaldson *et al.*2013b).

Sample preparation and possible reference materials for the safety evaluation of medical devices is described in ISO 10993-12:2012. Although this standard does not specifically address nanomaterials, it provides general information on sample preparation from solid materials. In addition, the nanomaterials themselves, e.g. when provided as powder of in liquid dispersion, may also be used in the assays for safety evaluation. For the sample preparation and dosing of nanomaterials, the OECD has prepared a Guidance document (OECD 2012).

### 3.7.3 Toxicity testing methods

**Cytotoxicity**
The ISO 10993 – 5:2009 describes test methods to assess the *in vitro* cytotoxicity of medical devices. In addition, a standard is currently under preparation for an *in vitro* cytotoxicity assay specifically dedicated to nanomaterials (ISO/AWI 19007 Modified MTS assay for measuring the effect of nanoparticles on cell viability, ISO, Geneva, Switzerland).

Driven by European politics on animal welfare, there are continuous efforts to find *in vitro* alternative methods to *in vivo* testing on animals. However, at the moment there are no validated *in vitro* methods for hazard assessment of both chemicals and nanomaterials, *In vitro* tests may be useful for screening purposes, for indicating potential toxicity of a nanomaterial and to elucidate possible modes of action (Nel *et al.*2013b), providing pointers for further toxicological investigations. For example, *in vitro* tests may indicate the likelihood of generation of reactive oxygen species (Xia *et al.*2008), which may provide an alert for potential toxic effects via the induction of oxidative stress and activation of inflammatory and proliferative pathways (Unfried *et al.*2008, Donaldson *et al.*2010, 2013b).

Considering acute toxicity testing for appropriate classification, a number of cytotoxicity assays have been proposed. Recently a major effort has been undertaken (AcuteTox project - www.acutox.org) to create an integrated testing strategy to replace the animal testing for predicting human acute oral systemic toxicity which is based exclusively on *in vitro* and *in silico* methods. The 3T3/NRU assay was indicated as a first step in a tiered testing strategy suitable for the identification of unclassified substances (LD50 > 2000 mg/kg). The assay has been endorsed by the European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM) for screening of industrial chemicals for acute toxicity testing (https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-recommendations/files-3t3/ReqNo_JRC79556_lbna25946enn.pdf).
However, nanomaterials were not included in the selected tested substances and therefore, the use of such test should be evaluated on a case-by-case basis.

**Acute toxicity**
Acute toxicity testing for medical devices is part of the ISO 10993-11: 2006 standard dealing with determination of systemic toxicity of medical devices (ISO 2006). An acute toxicity study might be an initial step in establishing a dosage regimen in subacute/subchronic and other studies and may provide information on the mode of toxic action of a substance by the intended clinical exposure route. For medical devices, the test sample preparation, consisting in general of both a hydrophilic and a lipophilic

extract of the material or the medical device, is presented in ISO 10993-12: 2012 (ISO 2012). However, to obtain an indication of the toxicity of a nanomaterial, a dispersion of the nanomaterial itself as it is used in the medical device may also be considered. Other information on the performance of an acute toxicity test in addition to ISO 10993-11:2006 can be found as follows: for acute oral toxicity testing using the fixed dose method [EC B.1 bis, OECD 420], the acute toxic class method [EC B.1 tris, OECD 423], or the up-and-down procedure [OECD 425]. The acute toxic class method by the inhalation route is described in OECD 403 and 436, and the *In vivo* acute dermal toxicity assay is described in EC B.3 and OECD 402 and 404.

**Irritation activity**
The ISO – 10993-10:2010 describes test methods to assess the potential to produce irritation (and delayed-type hypersensitivity) of medical devices and their components after topical skin application. In addition, ISO 10993-10:2010 describes intradermal irritation tests for medical devices used as implants or transdermally. Specific irritation tests are described in Annex B including eye irritation test, oral mucosal irritation test, and rectal, penile and vaginal irritation tests.
For chemicals, the determination of irritation/corrosivity an *in vivo* method is used based on Draize test as described in EC B.5, and OECD 405.

The following five validated *in vitro* alternatives are available (Regulation (EC) No 440/2008) for skin corrosion assessment of chemicals:

a) TER test (rat skin transcutaneous electrical resistance test) [EC B.40, OECD 430]
b) EpiSkin™, EpiDerm™, SkinEthic™, EST-1000 [EC B.40bis, OECD 431]

Three of them, namely EpiSkin™, Modified Epiderm™Skin Irritation Test (SIT) and SkinEthic™ Reconstructed Human Epidermis (RHE) are validated for skin irritation assessment for chemicals (OECD 439).

No specific validation of the *in vitro* alternative tests has been performed for medical devices and/or nanomaterials, although there is no clear scientific basis against the use of these methods for nanomaterials.

The assessment *in vivo* of eye irritancy or corrosiveness on substances is based on the result of the classical Draize *in vivo* eye irritation test on rabbits according to EC B.5, and OECD 405 test guideline. There are alternative methods available replacing this test: the Bovine Cornea Opacity Permeability (BCOP) [OECD 437], the Isolated Chicken Eye (ICE) [OECD 438], and an *in vitro* cell assay (OECD 460). These assays, although using animal eyes, are considered alternatives, since they are obtained from animal slaughterhouses. They are able to discriminate corrosive and severe eye irritants, but fail to distinguish mild from non-irritants.

These assays were developed for the evaluation of skin irritation of chemical substances. It is yet unknown whether they can be used also for the skin irritation activity of medical device extracts and/or nanomaterials. The ICE test is not suitable for solid samples. The assays can probably be also used for nanomaterials, but there is no validation to date: they may provide supporting evidence.

It is possible that some insoluble particulate materials can induce eye irritation not only chemically, but may also mechanically interfere with the eye tissue or the cell.

**Delayed-type hypersensitivity**
ISO – 10993-10:2010 describes test methods to assess the potential to induce delayed-type hypersensitivity for medical devices and their components. Three *in vivo* methods are described, two using guinea pigs and one using mice, for assessing skin sensitisation potential. The murine local lymph node assay (LLNA) in its three versions is the preferred method in view of animal welfare (OECD 429, 442A and 442B). The two guinea pig

assays are the Magnusson Kligman Guinea Pig Maximisation Test (GPMT), as described in EC B.6, OECD 406, and ISO 10993-10:2010, and the Buehler test (EC B.6, OECD 406, ISO 10993-10:2010).

Due to the larger surface area of particles, nanomaterials may be regarded as potential allergic chemicals through their adjuvant capacity and complex formation with cell proteins (Larsen *et al.*2010, Lee *et al.*2011).

The above described standard tests for skin sensitisation have not been specifically evaluated for the testing of nanomaterials. A significant difference exists between the LLNA and the Buehler test that both involve application of the test compounds (e.g. nanomaterials) on the surface of the skin, and the GPMT that involves intradermal application. The LLNA is used to verify sensitisation of nanomaterials, but there are no positive responses (Lee *et al.*2011). In addition, the LLNA is used to verify whether nanomaterials can potentiate the level of sensitisation of known sensitisers (Lee *et al.*2011). The value of both tests in Lee *et al.* (2011) was challenged because dermal penetration was not assessed. Currently, limited experimental data is available on nanomaterials tested using GPMT. Negative results were reported for ZnO using a modified GPMT with topical application on a FCA treated skin (Jang *et al.*2012, Park *et al.*2013).

Based on the current knowledge, it is not possible to rely on the use of one specific test method for nanomaterials. The use of LLNA and/or Buehler test will probably not result in sensitisation, due to possible low skin penetration of nanomaterials. In view of the intradermal application, the GPMT is currently the most relevant test for detecting possible sensitisation activity of nanomaterials, although the intradermal induction phase is followed by a topical induction phase and topical challenge in the intact skin.
Importantly, these tests only identify the hazard for delayed type hypersensitivity; for acute hypersensitivity mediated by immunoglobulin-E, no assays are currently available. For certain iron nanoformulations after intravenous administration, systemic allergic responses were observed as reported in an assessment report by EMA (EMA 2013).

**Genotoxicity**
ISO 10993-1 indicates considerations for identifying when the potential for genotoxicity is a relevant hazard. In general the testing for genotoxicity is not necessary for medical devices, and components thereof, made only from non-genotoxic materials. This rule might also apply for nanomaterials. However, if the genotoxicity of the ingredients including nanomaterials is unknown, genotoxicity testing is necessary. ISO 10993–3:2003 describes tests for genotoxicity (carcinogenicity and reproductive toxicology). A recent review on genotoxicity testing concluded that genotoxicity testing should also consider other potential toxic effects in the assays (Magdolenova *et al.*2014). "Many studies, both *in vitro* and *in vivo*, show positive effects most likely due to the use of concentrations that are not relevant to possible environmental exposure. In many studies a demonstration of genotoxicity simply reflects cytotoxicity, as excessively high concentrations are used. Thus, cytotoxicity should be an integral part of genotoxicity testing to avoid false-positive results" (Magdolenova *et al.*2014).

It should be noted that for all genotoxicity assays it is important to establish exposure of the target cells and/or organs to the nanomaterials tested. In addition, besides direct genotoxicity, other mechanisms of toxicity may also result in DNA damage and induce indirectly genotoxicity, notably chronic inflammation (Kundu and Surh 2008. Donaldson *et al.*2011).

***In vitro* genotoxicity testing**
In selecting a suitable battery of *in vitro* genotoxicity tests, the three critical genotoxicity endpoints (gene mutation, structural and numerical chromosome aberrations) should also be considered for nanomaterials.

Although a bacterial reverse mutation assay (Ames test, OECD 471) is a reliable genotoxicity screen for the analysis of chemicals, it does not appear to be suitable for the assessment of nanomaterials. This might be related to the degree of uptake by the bacterial cells, which is likely to be less than in human cells for two reasons. Firstly, prokaryotes cannot perform endocytosis and secondly, their cell wall forms a barrier against simple diffusion of nanomaterials (particularly those in agglomerated form) into the bacterial cell – this lack of uptake could potentially lead to false negative results. Therefore, the Ames test is unlikely to be a suitable general *in vitro* genotoxicity screening test for nanomaterials, although recently uptake of nanomaterials was observed in the Ames test (Clift *et al.*2013). Additionally, modifications to the technique may need to be considered to promote uptake of nanomaterials into the Ames test bacteria to reduce the potential for false negative results (Landsiedel *et al.*2009, Doak *et al.*. 2012, Magdalenova *et al.*, 2012, 2014).

The following *in vitro* tests seem to be the best options for testing nanomaterials:
1. A test for induction of gene mutations in mammalian cells (e.g. the mouse lymphoma *tk* assay with colony sizing and/or the CHO/HGPRT mutation assay) (OECD 476)
2. An *in vitro* micronucleus assay (OECD 487) or a chromosome aberration test (OECD 473)
3. An *in vitro* Comet assay

There may be circumstances under which it may be justified to deviate from the above-mentioned core set, e.g. when there is a need to test the nanomaterial in a matrix that cannot be added *in vitro*. In such cases, a scientific justification should be provided and additional types of considerations or *in vivo* studies may be needed. In certain instances, e.g. soluble, very small, inducing reactive oxygen species nanomaterials, a bacterial reverse mutation test might still be informative.

For all *in vitro* tests, uptake of the nanomaterial in either bacteria and cells should be demonstrated to indicate potential DNA exposure to the nanomaterial under investigation.

Caution is needed with the micronucleus test when nanomaterials are tested. Cytochalasin B, which is often used to inhibit cytokinesis, may also inhibit endocytosis, and hence has been suggested to lead to false negative outcomes with nanoparticles (Landsiedel *et al.*2009). Especially when Cytochalasin B and the nanomaterials are added to the test system simultaneously at the start of the experiment. This might be avoided by not adding the Cytochalasin B simultaneously with the nanomaterials, but after the start of the incubation (e.g. at 6 hours after adding the nanomaterials to the cells).

Moreover, for several types of nanoparticles (e.g. titanium dioxide, multi-walled carbon nanotubes), the microscopic evaluation of cytokinesis-block proliferation index and micronucleus identification was found to be rather difficult at high testing concentrations due to the abundant presence of nanomaterials in the cells (Corradi *et al.*2012). This problem might be (partly) solved, for example, by histological staining with fluorescent labelled DNA probes that reduces the risk of falsely identifying nanoparticle aggregates as micronuclei fragments in the micronucleus test (Magdolenova *et al.*2014). In the comet assay it was shown that nanomaterials tested did not interact with endonucleases used for detection of DNA breaks (Magdolenova *et al.*2012).

***In vivo* genotoxicity testing**

Unless it can be adequately demonstrated that positive *in vitro* findings are not relevant for the *in vivo* situation or if it is impossible to test the nanomaterial *in vitro*, *in vivo* testing is necessary (Eastmond *et al.*2009). Before embarking on any necessary follow-up, other relevant data on the substance, such as information about chemical reactivity

(which might predispose the site of contact effects), bioavailability, metabolism, toxicokinetics, and any target organ specificity should be considered.
*In vivo* genotoxicity tests should relate to the genotoxic endpoint(s) identified as positive *in vitro* and to appropriate target organs or tissues. Evidence, either from the test itself or from other toxicokinetic or repeated-dose toxicological studies, that the target tissue(s) have been exposed to the test substance and/or its metabolites is essential for interpretation of negative results. The choice of the appropriate *in vivo* genotoxicity test(s) requires expert judgement based on all available information, to be applied case-by-case. Any of the following *in vivo* tests may be suitable

- an *in vivo* micronucleus test (OECD 474).
- an *in vivo* mammalian bone marrow chromosome aberration test (OECD 475)
- an *in vivo* mammalian spermatogonial chromosome aberration test (OECD 483)
- a transgenic rodent gene mutation assay (OECD 488)
- an *in vivo* Comet assay (OECD 489)

However, these guidelines were developed for testing chemicals, and their suitability for nanomaterials testing should not be taken for granted because their distinct physicochemical properties can seriously influence their interactions with DNA (Dusinska *et al.* 2009; Warheit and Donner 2010, Magdalenova *et al.* 2012, 2014). In addition, the transgenic rodent mutation assay was identified as adequate to detect chemically-induced gene mutations with some limitations in all tissues but there may be practical limitations when performing the assay (ECHA 2012).

**Haemocompatibility**
The ISO 10993-4:2002 (and its amendment 10993-4:2002/Amd 1:2006) standard is applicable to devices that contact the circulating blood and serve as a conduit into the vascular system. Medical devices that need to be evaluated for their blood compatibility include external communicating devices that have an indirect blood contact, external communicating devices directly in contact with circulating blood, and implant devices that are placed largely or entirely within the vascular system.

Most tests for haemocompatibility according to ISO 10993-4:2002 are based on direct contact between a surface and whole blood or components of blood. Thus materials with nano-structures on their surface can be directly evaluated using the same methods described in the 10993-4 standard on selection of tests for interactions with blood.
For nano-materials in general or in particular form there are no established tests available today. One of the tests in the 10993-4 standard, the haemolysis test, is based on the testing of extracts and a suspension of nanoparticles could thus be used for testing.

When contact with blood is possible, especially for free nanomaterials/nanoparticles a potential interaction with phagocytic cells, e.g. polymorphonuclear cells and monocytes, has to be carefully considered. The nanoparticles may be presented with different surface properties and in different aggregate forms depending on which medium they are suspended. These factors are critical for the interaction with phagocytic cells.

No standards are currently available for the evaluation of particle and especially nano-particle interaction with phagocytic cells. Although in many *in vitro* tests, phagocytic macrophages are used as target cell. One possible way to indirectly evaluate the haemocompatibility of particulate nanomaterial is to inject a suspension of nano-particles into the vasculature and evaluate the distribution as well as any local and systemic signs of adverse events like vascular damage, activation of complement, activation of the coagulation cascade or activation of platelets. Methods for testing of activation of complement, coagulation cascade and activation of platelets are described in the 10993-4 standard.

In addition, new techniques (e.g. microfluidics) may be needed to evaluate the interaction of (nano)particles with endothelial cells and/or the vasculature (Santos-Martinez *et al.* 2011, Samuel *et al.* 2012).

**Repeated- dose toxicity**

The ISO 10993-11:2006 describes specifically for medical devices tests for repeated dose toxicity appropriate for the route and duration of exposure. Repeated dose toxicity testing for chemicals is described in various OECD test guidelines (407, 408, 409, 411, 412, 413, 415, 416, 422, 443, 451, 452, 453).

ISO 10993-11:2006 addresses the evaluation of generalised systemic toxicity, not specific target organ or organ system toxicity, even though these effects may result from the systemic absorption and distribution of substances released from medical devices. Because of the broad range of substances used for the production of medical devices and intended uses, this part of ISO 10993 is not overly prescriptive. Whilst it addresses specific methodological aspects considered in the design of systemic toxicity tests, proper study design for the evaluation of nanomaterials must be uniquely tailored to the nature of the nanomaterials present in a medical device and its intended clinical application or use.

Whenever possible, the nanomaterials in medical devices should be tested in a form representative of its "ready to use" state and applied under most adequate conditions in which it is to be used. Testing should be performed on nanomaterials obtained from the final product and/or representative component samples of the final product.

Preferably the repeated dose toxicity studies should be performed based on the location of the potential exposure i.e. the site of the use of the medical device, and the knowledge on the toxicokinetics of the released nanomaterials. However, due to practical reasons, most of the repeated-dose toxicity testing is performed using oral route. The administration of test material in the *in vivo* oral toxicity studies could be done by adding the nanomaterial to the animal feed, to the drinking water, or by gavage. In this case, information should be available on the occurrence of potential differences in the bioavailability of the nanomaterial depending on the route of exposure as was demonstrated for Au nanoparticles for intratracheal and intravenous administration (Oberdörster 2010, Semmler-Behnke *et al.* 2008).

For administration, the nanomaterial should ideally be homogeneously blended into the feed matrix or stably and uniformly dispersed in the drinking water or gavage vehicle. The stability and physicochemical characteristics of the nanomaterial in the vehicle should be determined. Possible interactions with the administration vehicle should also be determined in advance before choosing the way of exposure to nanomaterials.

There may be limitations on the amounts of nanomaterial that can be administered, because it may agglomerate in the drinking water or gavage vehicle, or they may already be blended as agglomerated powder into the feed, which in addition may not be uniformly mixed within the food matrix. The administration of the test material requires careful control and dynamic characterisation of tested nanomaterials in either the liquid or the feed matrix. For example, a nanomaterial in liquid may adsorb into the walls of the drinking vessel and therefore no longer be available, i.e. there will be no exposure.

To overcome some of the obstacles mentioned above, a nanomaterial can be applied by gavage, aiming for the nanomaterial to be dispersed, characterised and administered under well-defined conditions. However, application by gavage is not likely to be representative of the lower concentrations delivered over time from nanomaterial administered via feed. Gavage provides a bolus of the material at a given time that may or may not mix with the gastrointestinal fluids, which might result in a higher local concentration and increased quantity of absorbed material due to the nanomaterial being in the form of a single, large dose and the lack of co-ingestion of dietary components to which nanomaterial can easily bind.

In any of the oral administrations mentioned above, the passage through the acid environment of the stomach and mixing with the chyme in the gut may affect the nanomaterial. Consideration of the potential for time dependent dissolution/ degradation is essential, as well as physicochemical nanomaterial modifications such as agglomeration and surface modifications by proteins and biomolecules. The fate of nanoparticles in the GI-tract can be investigated in *in vitro* models using simulated fluids or more complex systems (Minekus *et al.* 1999, Oomen *et al.* 2004) and has also been applied to nanoparticles (Rogers *et al.* 2012, Peters *et al.* 2012, Mwilu *et al.* 2013).

However, the systemic availability of nanomaterials after oral administration may be limited (see 3.6.4). Initial toxicokinetic studies might indicate whether oral administration is a proper method for identifying potential systemic toxicity. Other routes for evaluating systemic toxicity may also need to be considered (e.g. intravenous, subcutaneous administration) depending on the use of the medical device.

**Implantation**
At present, there are no accepted or validated methods for biological evaluation of implanted nanomaterials. However, some Guidance can be found in ISO 19003-6:2007. The test methods apply to a wide range of materials such as solid and non-absorbable, absorbable, non-solid such as porous materials, liquids, gels, pastes and particulates.
The test methods may also be applied to medical devices that are intended to be used topically in clinical indications when the surface or lining may have been breached in order to evaluate local tissue responses.

The local effects are evaluated by a comparison of the tissue response caused by a test specimen to that caused by control materials used in medical devices of which the clinical acceptability and biocompatibility characteristics have been established. The objective of the test methods is to characterise the history and evolution of the tissue response after implantation of a medical device/biomaterial including final integration or absorption of the material. In particular for absorbable materials the degradation characteristics of the material and the resulting tissue response should be determined. All materials will provoke an inflammatory response when implanted. It is the extent and seriousness of this local inflammatory reaction that indicates whether this reaction should be considered adverse. For non-degradable materials, a steady state on the tissue response is generally obtained after 12 weeks, while for absorbable materials this depends on the rate of absorption that may be shorter or much longer than 12 weeks.

ISO 10993-6:2007 on implantation testing does not deal with systemic toxicity, carcinogenicity, teratogenicity or mutagenicity. However, the long-term implantation studies intended for evaluation of local biological effects may provide insight into some of these properties. Systemic toxicity studies conducted by implantation (ISO 10993-11:2006) may satisfy the requirements of this part of ISO 10993-6. When conducting combined studies for evaluating local effects and systemic effects, the requirements of both standards needs to be fulfilled.

It can be reasonably anticipated that the tissue response to absorbable implant materials will be different from the tissue response found in non-absorbable (durable) implants. The assumption should be one of continuous interaction of the degrading material with the surrounding tissue, accompanied with an ongoing presence of a degradation-rate-dependent tissue response. Such a response may vary over time and may (or may not) be histologically detectable dependent upon the composition and manufacturing of the materials, the rate of degradation, the time post-implantation, and the tissue within which the implant resides. This tissue response should resolve and normal morphology be restored as the degrading material is absorbed into the surrounding tissue.

To properly evaluate an absorbable implant and its degradation products, local tissue responses may need to be assessed at more and different study intervals than those typical for non-absorbable materials. The provisions in ISO 10993-6 (Annex A, General considerations regarding implantation periods and tissue responses to absorbable materials) are also applicable to the evaluation of the local effects of absorbable materials used as carriers for drug release, scaffolds for tissue-engineered medical products, or surface coatings for non-absorbable implants.

The particles may have a local effect at the site of the implant but may also show migration for example to the draining lymph nodes. Local effects are limited to the site of the implantation (or use of the medical device) and depend on that localisation. An example of such a local effect is wear of joint prostheses leading to particle accumulation in synovial fluid and synovial tissues. Biological effects are greatly influenced, whether the particles are deposited in subcutaneous tissue, intraperitoneally or into the blood.

**Chronic toxicity/carcinogenicity**
ISO 10993–3:2003 describes tests for genotoxicity, carcinogenicity and reproductive toxicity. The decision to perform a carcinogenicity test that usually lasts for two years, should be justified on the basis of the potential exposure arising from the use of the medical device, nanomaterials and or their extracts. However, in practice, it is rarely considered applicable to investigate carcinogenicity, because of the already existing knowledge on the material used for a medical device. The most common *in vivo* tests to assess the carcinogenic potential of chemicals are:
a) Carcinogenicity test [EC B.32, OECD 451]
b) Combined chronic toxicity/ carcinogenicity test [EC B.33, OECD 453], but no indication about their suitability for nanoparticles has been provided so far. Therefore, the use of such tests should be evaluated on a case-by-case basis.

**Reproductive and developmental toxicity**
Before a decision to perform reproductive and developmental toxicity tests is made, ISO 10993-1:2009 and ISO 10993-3:2003 should be taken into consideration.

There is no need for reproductive toxicity testing of resorbable medical devices or medical devices containing leachable nanomaterials/nanoparticles if there are adequate and reassuring data from absorption, distribution, metabolism and excretion (ADME) studies indicating that the neither the test item nor its metabolites are distributed and therefore do not reach the reproductive organs/targets, or if there is no reproductive toxicity of all components in extracts of medical devices.

In the absence of evidence to rule out reproductive/developmental risks, testing should be considered. This may include tests on the following medical devices containing nanomaterials:

a) prolonged or permanent-contact medical devices likely to come into direct contact with reproductive tissues, embryos or foetus
b) energy-depositing medical devices
c) resorbable or containing leachable nanomaterials/nanoparticles

If testing is required, this should start with OECD 421 (Reproduction/Developmental Toxicity Screening Test) to provide initial information on possible effects on reproduction and/or development. Positive results with tests are useful for initial hazard assessment and contribute to decisions with respect to the necessity for timing of additional tests. If additional tests are considered necessary, in view of the outcome of the screening test, they should be performed in accordance with OECD 414 (Prenatal Developmental Toxicity Study), OECD 415 (One-Generation Reproduction Toxicity Study), OECD 416 (Two-Generation Reproduction Toxicity Study) or OECD 422 (Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test), as appropriate. No

indication is available on the suitability of these tests designed for chemicals to assess the reproductive toxicity potential of nanoparticles. Therefore, the use of such methodologies should be evaluated on a case-by-case basis.

More recently, test guideline OECD 443 was published on the so-called extended one generation reproductive toxicity study, which combines several endpoints including reproductive/developmental endpoints, neurodevelopmental and immune developmental endpoints.

Methods for embryotoxicity testing are likely to be applicable to nanomaterials, provided that typical nanomaterial related issues such as dispersion/ aggregation, adsorption, stability and distribution into the tissue are taken into consideration. In an *in vitro* embryonal stem cell assay, which was used for research purposes only, effects on cardiomyocyte development were observed for silica nanoparticles (Park *et al.* 2009).

Assessment of effects on the first generation (F1) or even second generation (F2) should be made in accordance with OECD 414, OECD 415, OECD 416, OECD 421, OECD 422 or OECDE 443. As the OECD guidelines were not intended for nanomaterials/nanoparticles in medical devices, the following modifications should be considered: dose (in the case of energy-depositing devices), route of application (implant, parenteral, other), extraction media (aqueous and non aqueous extracts) or exposure time.
It is not recommended to use methods of exposure that for some reason could affect prenatal development. For example, intraperitoneal administration may cause the tested nanomaterials/nanoparticles to be directly injected in the uterus itself or pass through the wall of the uterus and directly affect the developing embryos/foetuses. Inhalation exposure "nose only" does not seem to be appropriate for pregnant females due to the fact that an animal is present under forced, stressful conditions, pretty tight restrainer for about 6h/day without access to feed and water.

In the developmental toxicity study, one should be aware of possible exposure to offspring via breast milk (Melnik *et al.* 2013) The presence and concentration of nanomaterials/nanoparticles in the milk of lactating animals should be measured.

## 3.8. Evaluation of nanomaterials used in medical devices

The evaluation of the risk of chemicals leaching from a medical device is described in EN ISO 10993-17:2002. The methodology for the evaluation of allowable limits for chemicals may also be applied to nanomaterials. In this standard, the estimated exposure needs to be compared with the toxicity information. In addition, the benefit for the patient needs also to be considered in the evaluation of medical devices.

In addition, for the selection of safety evaluation assays as presented for medical devices in ISO 10993-1:2009, specific testing for the nanomaterials used in a medical device may be necessary. The testing to be performed is determined similarly to ISO 10993-1 but now based on the potential for release of the nanomaterials from the device and the duration of exposure. According to ISO 10993-1, the need for testing for hazard identification is based on the type of medical device, type of contact and duration of exposure. A schedule is proposed in Table 4.

**Table 4: Framework for specific nanomaterial toxicity testing based on potential release (exposure) of nanomaterials from medical devices.**

| Testing proposed | Non-invasive short term use | Non-invasive long term use | Invasive short term use | Invasive long term use |
|---|---|---|---|---|
| Low exposure | Phys: chem data | Phys: chem data | Phys: chem data | Phys: chem data |
| | Cytotoxicity *in vitro* | Cytotoxicity *in vitro* | Cytotoxicity *in vitro* | Cytotoxicity *in vitro* |
| | Irritancy *in vitro* | Irritancy *in vitro* | Irritancy *in vitro* | Irritancy *in vitro* |
| | Hypersensitivity | Hypersensitivity | Hypersensitivity | Hypersensitivity |
| | | Genotoxicity *in vitro* | | Genotoxicity *in vitro* |
| | | | | General Immuno toxicity testing |
| Medium exposure Additional tests | | Genotoxicity *in vivo* | Other *in vitro* plus *in silico* testing* | 28/90 day *in vivo* toxicity test |
| | | Immuno toxicity at location site | Genotoxicity *in vitro* and *in vivo* | *In vitro* and *in vivo* (repeated dose) genotoxicity testing |
| | | Persistence /accumulation studies at location site only | | ADME including persistence /accumulation studies |
| High exposure Additional tests | Selected *in vivo* acute toxicity tests focussed on location site(s) | Selected *in vivo* chronic toxicity tests focussed on location site(s) | *In vivo* acute toxicity tests | *In vivo* chronic toxicity tests may include reprotox depending on patient group. |

*See also EURL-ECVAM database (http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/validation-regulatory-acceptance/)

Phys: chem indicates data from physicochemical characterisation of the nanomaterials.

The schedule presented in Table 4 should not be considered as a checklist of assays to be performed, but is intended as a Guidance for which assays have to be considered (in line with ISO 10993-1:2009) for the biological evaluation of a medical device containing nanomaterials. In some cases, specific studies may not be necessary, but a scientifically based and sound justification should be provided.

### 3.8.1. Non-invasive surface contacting medical devices

This category applies to devices that contact intact skin and breached or compromised surface (ISO 10993-1:2009)

Surface contacting medical devices will interact locally as long as the skin is un-breached. There is little or no evidence that nanoparticles will penetrate the natural skin, therefore

potential for internal systemic exposure is low or negligible regardless the type of application (Table 1). The local effects should be evaluated, e.g. cytotoxicity and irritation using the same principles as medical devices without nanomaterial components. However, established methods for the evaluation of sensitisation potency of nanomaterials are currently not available.

### 3.8.2. Invasive surface contacting medical devices

If there is a concern that the barrier properties of the skin are changed by a wound or an inflammatory process, the possibility that nanoparticles may penetrate and become deposited locally and migrate to other localisations should be considered. This may be done by investigating the actual penetration through compromised skin in an animal experimental model. However, such experimental models are difficult to establish and validate, and a better approach may be to investigate the effects of intradermally or subcutaneously introduced particles. This may follow the protocol of the already established intracutaneous irritation test as described in ISO 10993-10:2010, but extended to include histological evaluation of draining lymph nodes.

When a medical device containing nanomaterial is in contact with breached or compromised skin and nanomaterials are released, additional testing for systemic toxicity should be considered including genotoxicity testing independent of the contact duration time. A suitable battery of *in vitro* genotoxicity tests addressing three critical genotoxicity endpoints (gene mutation, structural and numerical chromosome aberrations) should be considered.

### 3.8.3. Invasive external communicating medical devices

This category applies to devices that can contact circulating blood at one point and serve as a conduit for entry into the vascular system (indirectly), circulating blood directly and that contact tissue, bone, pulp/dentin (ISO 10993-1:2009). Invasive external communicating medical devices may contain nanomaterials that may be released after material degradation or may be present as nano-size structures on its surface. Dialysis and oxygenating equipment are also included in this category.

It is of vital importance to consider the type of tissue that may be exposed. The effect on draining lymph nodes or other organs that may be reached after particle migration should be investigated. The presence of nanoparticles in tissue should be investigated using the appropriate identification techniques (e.g. ICP-MS, electron microscopy, fluorescent dye labelling), and whenever possible a quantitation should be performed.

### 3.8.4. Invasive implantable medical devices

An invasive medical device is defined as a device, which, in whole or in part, penetrates inside the body, either through a body orifice or through the surface of the body (Directive 93/42/EEC). This includes implant devices that contact principally blood, tissue and bone (ISO 10993-1:2009). Medical devices applied through body orifices coming into contact with mucosal membranes are also considered invasive medical devices. In general testing of this type of medical devices is performed according to ISO 10993-1, thus depending on the type of tissue contact and the duration of the contact. For nanomaterials used in medical devices, a similar approach should be considered, although special emphasis should be the potential release of the nanomaterials from the devices. Similar to invasive external communicating devices, local particle release should

be considered and possible effects on draining lymph nodes. Supporting data, if available, on the toxicological evaluation of nanomaterial ingredients may be used in the safety evaluation of medical devices. Depending on the release, the safety evaluation of the nanomaterial itself might be considered, taking into consideration the intended use of the medical device in which the nanomaterial is used.

### 3.8.5. Specific types of medical devices

For **wound care materials,** specific considerations apply. In some wound dressings, nanosilver is used for its antibacterial activity (Wijnhoven *et al.* 2009, SCENIHR 2013). They are also used on breached and compromised skin. Therefore, direct contact with subcutaneous tissues including blood is possible. When there is a considerable release of the nanomaterials used in wound dressings, systemic exposure may also be possible. Thus, a more extensive risk evaluation of the nanomaterial component should be considered (SCENIHR 2013).

**Uncured dental and bone fillers** and cements may contain and even consist of free nanoparticles. Cements and dental fillers are typically cured *in situ* resulting in a solid mass of (bio)material. During the application of dental materials and also during surface treatment, e.g. polishing, nanoparticle exposure may occur. Depending on the application site (dental use in the oral cavity or orthopaedic use of bone cement), internal exposure to nanoparticles is possible. For dental materials, lung exposure should also be considered (see below). This specific potential internal exposure should be considered in the risk evaluation of such materials.

In exceptional circumstances, **injectable nanomaterials** might be classified as Devices rather than Medicinal Products. For injectable nanomaterials, there is the potential of internal exposure which may be high depending on the dose administered. For these applications, extensive distribution studies are warranted. However, the extent of the systemic exposure is dependent on the injection site. For subcutaneous injections, the distribution via the local draining lymph nodes should be evaluated. However, further distribution should be investigated, because it cannot be assumed that further distribution beyond the local lymph node does not occur. For other injections, systemic exposure is likely, or certain, e.g. after intravenous administration and extensive toxicokinetic and systemic toxicity studies are warranted. EMA (London, UK) has published several Guidance documents on injectable nanomedicines (http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_000564.jsp&mid=WC0b01ac05806403e0).

**Medical devices resulting in respiratory tract exposure**. When nanomaterials are used in medical devices applied in the respiratory tract, the possibility for lung exposure exists. The handling, e.g. polishing, of dental materials may also result in respiratory tract exposure to particles (Van Landuyt *et al.* 2012, 2014; Bogdan *et al.* 2014). Inhalation of various particles was shown consistently to induce local adverse effects in the lung. Inhaled particles reach different target compartments of the lung tissues depending on their size, e.g. particles about below 50 nm in diameter seem to be most effective in reaching the pulmonary alveoli (ICRP 1994, Cassee *et al.* 2002). In addition, regarding lung exposure of nanomaterials, effects on the cardiovascular system should also be considered (Donaldson *et al.* 2013a).

**Combination products.** A specific subgroup may be the so-called "combination products", where devices incorporate a substance as an integral part which, if used separately, may be considered to be a medicinal product. The safety, quality and usefulness of the medicinal substance must be verified by analogy with the methods required by in Directive 2001/83/EC (Medicinal Products for Human Use) concerning the testing of medicinal products.

### 3.8.6. Conclusions

Non-invasive medical devices containing nanomaterials in most instances, with the exception of local reactions at the site of contact, do not pose an additional risk compared to non-invasive medical devices that do not contain nanomaterials and may be evaluated using the same methodology.

For invasive medical devices containing nanomaterials, including surface contacting devices in contact with breached skin or mucosa, the same principles for toxicity testing apply as for medical devices that do not contain nanomaterials. However, the biological effects of nanoparticles that are introduced or formed should be investigated both for local effects at the site of application and at possible distribution organs after migration, especially draining lymph nodes. In the safety evaluation, the potential release, accumulation, and persistence of the nanomaterials in the tissues requires further testing. In this context, the possible dissolution/degradation of the nanomaterials should also be considered.

All safety evaluations should consider the potential specific physicochemical properties of these nanomaterials, especially those medical devices that consist of free nanomaterials. The biological effects of nanoparticles that are introduced should be investigated both at the site they are deposited and in possible target organs for migration, especially draining lymph nodes.

In addition, the potential generation of nanosized particles due to wear-and-tear needs to be considered for all implant medical devices.

## 4. RISK EVALUATION

An estimation of the potential risk can be made based on the information obtained on nanomaterial characteristics, use as or in a medical device. The exposure may be considered as the outcome of the potential release from the medical device in the actual use conditions (exposure scenario) and the toxicokinetics of the nanomaterial (giving indication of the possible internal exposure). The risk can be estimated based on the potential exposure and the outcome of the safety testing according to ISO 10993-1:2009. Of major importance for the risk assessment is the possibility for release of the nanomaterial from the medical device. If particle release is not present, it is assumed that material and surface properties that may result in local reactions like inflammation and/or induction of allergy, and which may be related to particle reactivity, are adequately covered by the existing testing regimen as presented in ISO 10993-1:2009. Analogously, in the absence of any absorption, no systemic toxicity testing need to be carried out.

A phased approach to the risk assessment related to particle release is proposed below and is illustrated in Figure 2.

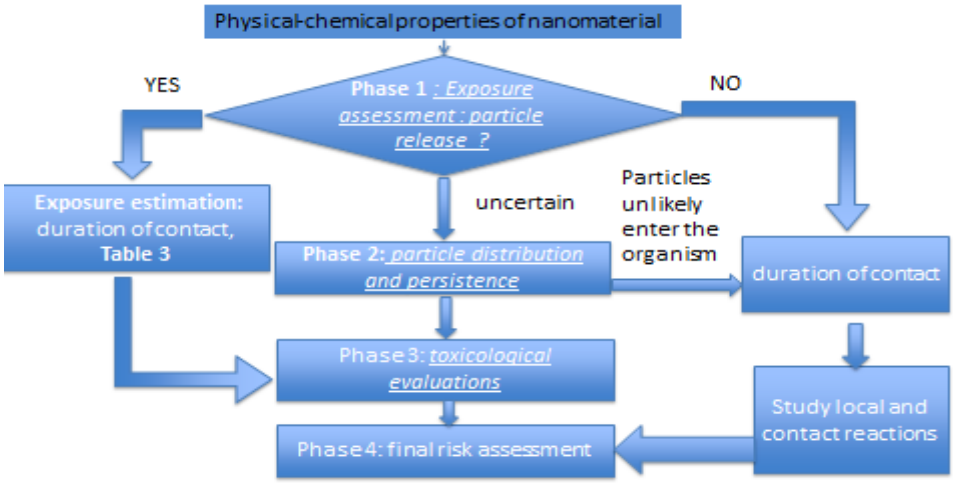


**Fig. 2**: Risk assessment of nanomaterials used in invasive medical devises: a phase approach

*Phase 1 Exposure assessment: particle release.*

The purpose of the first phase is to consider the likelihood that nanoparticles will be released to estimate potential exposure, either as an intrinsic property of the device or due to wear once implanted. If there is substantial evidence that the nanomaterials are embodied in the device or so well fixed that they will be retained in the device during insertion, period of use and removal, and that provided particles are not released as a consequence of wear, no further specific risk assessment regarding the nanoparticle component is required. It is important, however, that the relevant data of non-release are obtained under realistic worst case conditions.

For the exposure assessment, the information as presented in section 3.5.4 Table 3 can be used.

When there can be a release of nano-sized material in an amount sufficient to raise concern or when such an amount is unknown, then evaluation of the physicochemical properties of the released particles is necessary. It is essential that the particles studied in assays for the risk assessment are equivalent, in terms of both physical and chemical properties, as those that may be released *in situ*. Several scenarios could be identified for nanoparticle release including machining, weathering, washing, contact and incineration (Frogget *et al.* 2014). Identified debris consisted of particles from matrix alone, matrix particles with the nanomaterial embedded, the nanomaterials themselves or dissolved ionic forms of the added nanomaterial.

Physicochemical properties that should be considered include:

- Solubility in water. If solubility in water is prompt, no further consideration is needed in regard to the particulate nature of the released material though of course the potential adverse effects of the solubilised material will need to be considered further.
- Particle size distribution and shape. The mobility of particles and the effectiveness of the biological defence mechanisms to deal with them are affected by both the size and shape of the particles.
- Ability to agglomerate and dis-agglomerate. The ability for particles to combine and dissociate is also a factor that affects particle size. The larger the particle size in biological media the less likely will be the retention of the surface active properties that are associated with nanoparticles.
- Other characteristics dependent on the nanomaterial used like surface chemistry/composition (see also ISO TR 13014:2012).

The realistic worst case conditions for identifying the amount, estimated rate and number of released nanomaterials need to take into account the potential duration of contact of the medical device with the body. When exposure is expected/estimated due to nanoparticle release, further investigation is necessary.

If particle release does not occur, further evaluation may be limited mainly to investigate local reactions. If there is uncertainty regarding potential release of (nano)particles, a phase 2 assessment should be conducted.

*Phase 2 Exposure assessment: particle distribution and persistence*

*i) particle distribution*

The primary purpose of this phase is to identify the kinetics of the particles to address the toxicity testing needed in Phase 3 (below) based on potential exposure scenarios indicated above. It is self-evident that the absorption of particles released from non-invasive medical devices into the systemic circulation and/or location of the invasive device on/in the body and contact duration will have a major influence on the potential for distribution of the particles to other organs. A further consideration is the persistence/stability of the particles in the biological media into which they are released.

a) Non-invasive (skin)

The key issue is to identify the likelihood of penetration of the skin barrier. If negligible, then consider only the potential for effects at the topical site of application that need to be examined in the phase 3.

b) Invasive

Uptake of particles from the lung into the systemic circulation is more likely than from other external location sites. Therefore, the potential for released particles to reach the deep lung (alveolar region) and cross into the systemic circulation must be estimated. In

addition, local-term effects in the lung should be considered because most, if not all particles, induce lung inflammation.

Additionally, for other invasive devices, the distribution of released particles should be estimated, in particular, whether they remain at the site of the application of the device (if this can be demonstrated then the potential for accumulation needs to be given specific attention, but in principle only local toxic reactions need to be considered in the next phase). If there is a possibility of a more general distribution or if this possibility cannot be ruled out, a more in depth evaluation of the toxicokinetics is neccessary.

For external communicating devices, e.g. dialysis equipment the release of particles entering the systemic circulation has to be followed by appropriate toxicokinetic studies.

*ii) Particle persistence*

Both the number and the duration of particle presence in a specific tissue are important considerations affecting the likelihood of adverse effects occurring. Prolonged exposure of a tissue to released particles may arise for two reasons:

- Continuous release from the device
- Stability of the particles and their entrapment in a tissue or failure of clearance mechanisms.

The release due to the use of the device can be estimated based on short-term physicochemical studies as can the likely stability of the particles. Where release appears likely, *in vivo* animal studies may be necessary to achieve adequate characterisation of the internal exposure.

*Phase 3 Hazard assessment (toxicological evaluations).*

If particle release is not identified in phase 1 and/or phase 2, local effects of medical devices are assumed to be adequately covered by the existing testing regimen as presented in ISO 10993-1:2009.

Additional studies are necessary if there is release of particles. In deciding on the testing strategy, it is crucial to know the likely location (as identified in phase 2). If it is estimated from phase 2 that it is unlikely that particles that are released will enter the systemic circulation, then only tests to establish local effects are required. It is vital in such studies that the form of the nanoparticles used in the various studies is equal to that which is actually used and present (either released or created) in biological systems.

*a) Characterisation of local effects*

Of particular interest are the potential for:

- Irritation
- Immune reaction
- Cytotoxicity
- Genotoxicity
- Promotion of cell division

In principle some of these effects (e.g. genotoxicity) may be assessed initially in *in vitro* systems, as described in section 3.7.3, provided such test systems allow the penetration of the nanoparticles into the cell systems.

*b) Characterisation of systemic effects*

When there is exposure to particles in one or more tissues, a case-by-case approach needs to be adopted for which the approach of Table 4 in section 3.8 can be of help. Standard toxicity tests (see section 3.7.3) are suitable to assess the hazard although particular attention should be paid to the ability of the particles to concentrate in the

draining lymph nodes and other organs of the mononuclear phagocyte system. This may require some adaptation of traditional toxicity assessment protocols.

For acute exposures, only the scope of testing would be limited to acute studies unless there is a likelihood that a similar device will be used in the same patient on a number of occasions.

*Phase 4 Risk characterisation/risk assessment.*

Based on the possibility for exposure, the following categorisation (not intended to be quantitative) of the necessary risk assessment can be made (Table 5):

**Table 5: Framework for risk assessment of nanomaterials used in medical devices**

| **Release of nanoparticles** | **Non-invasive** | | **Invasive Lung** | | **Invasive Other** | |
|---|---|---|---|---|---|---|
| | Short exposure | Long exposure | Short exposure | Long exposure | Short exposure | Long exposure |
| Low/insignificant | N/VL* | L/F** | L | F | L | F |
| Medium | L/F | L/F | L/F | F | L/F | F |
| High | L/F | L/F | F | F | F | F |

*F=full assessment L=limited assessment VL =very limited or N= no further assessment *=limited assessment if it can be shown that penetration/distribution is very limited.*

*** Full assessment when absorption is indicated in toxicokinetic studies*

In cases where toxicity is induced by the nanomaterial used, particular attention must be given to the dose response relationship. The findings should be compared against the levels of particles found in the target organs (internal exposure) to evaluate the risk. The estimated risk may be compared to the risk from the use of comparable devices not incorporating nanomaterials, and assessed according to ISO 14971:2007. In addition to the estimated potential risk, ultimately also the potential benefit for the patient should be considered in the final benefit risk evaluation.

## 5. SUMMARY AND CONCLUSIONS

Many issues remain unresolved regarding nanomaterial safety and risk evaluation. However, a lot of information is already available, specifically on potential problems associated with the safety testing of nanomaterials. This information is included in the Guidance to raise awareness in the area of medical devices and to emphasize that the use of nanomaterials requires some specific considerations.

In the light of current knowledge, a case-by-case approach is necessary for risk evaluation of medical devices containing nanomaterials. A phased approach is proposed to avoid unnecessary testing.

In phase 1, an evaluation is needed of the potential for the device to release nanoparticles either directly or due to wear of the device during use. If the nanomaterial is fully embedded in the device, the consideration of potential wear resulting in the release of particles will probably be necessary. In addition, potential local effects of the device incorporating nanomaterials should be considered. For other devices containing nanoparticles, both release and wear considerations are necessary. If release of particles during the use of the medical device is deemed to be realistic, physicochemical tests are likely to be required to establish the nature of the released particles, the rate of release and factors likely to influence this. If as a result of these studies it is concluded that even under realistic worst case use conditions, particle release does not occur or will be negligible, further evaluation may be limited mainly to investigating local reactions.. When exposure is expected due to nanoparticle release, further evaluation of the risks is necessary.

In phase 2, the aim is to determine the distribution of the particles released and also their persistence potential. In the case of non-invasive devices, the potential of particles to enter the systemic circulation and thereby be distributed to various tissues is the prime consideration. If it is concluded that it is unlikely that the particles could enter the systemic circulation even under realistic worst case conditions of use, then only a very limited toxicity testing protocol is needed, which would be generally limited to local effects at the contact site.

For invasive devices, a more detailed study of the potential of the particles to access and remain in specific tissues is required by toxicokinetic studies. The findings from these studies will influence the choice of further toxicity testing methods.

In phase 3, the hazard is assessed by selecting toxicity tests that are relevant based on the nature of the observed exposure and potential persistent in specific organs. For some assays, evaluating potential hazards of nanomaterials adaptation of existing assays may be necessary.

In the future, as our knowledge of the properties of nanomaterials improves, it may be possible to predict the nature, distribution, tissue levels and potential persistence of the particles but this is unlikely to be possible in the near future.

The information gathered will give input for the final risk characterisation (phase 4). The estimated risk should be compared to the risk from the use of comparable devices not incorporating nanomaterials in judging the acceptability of the risk. In addition to the estimated potential risk, ultimately the potential benefit for the patient should also be considered in the final risk assessment.

In conclusion, the potential risk due to the use of nanomaterials in medical devices is mainly associated with the possibility for release of free nanoparticles from the device and to the duration of exposure. The potential release is dependent on the method of use of the nanomaterials, as free nanomaterial, nanomaterials fixed on surfaces or nanomaterials embedded in a matrix. In addition to particle release and potential effects of these particles, possible local effects at the site of application should also be considered. Importantly, wear-and-tear of a medical device may result in the generation

of nanosized particles even when the medical device does not contain nanomaterials. In addition, it is also possible to apply this Guidance for the safety evaluation and risk assessment of particles with a size larger than 100 nm.

## 6. CONSIDERATION OF THE RESPONSES RECEIVED DURING THE CONSULTATION PROCESS

A public consultation on this Opinion was opened on the website of the Scientific Committees from 18 July 2014 to 3 October 2014. Information about the public consultation was broadly communicated to national authorities, international organisations and other stakeholders.

Eleven organisations and companies participated in the public consultation, providing input to the main scientific questions (in total 110 contributions were received).

Each submission was carefully considered by the SCENIHR and the Working Group and consequently the scientific opinion has been revised to take account of relevant comments. The literature has been accordingly updated with relevant publications.

The scientific rationale and the opinion section were clarified and strengthened. However, a number of comments received addressed issues dealing with medicinal products and/or so-called borderline products that might be considered either as a medicinal product or as a medical device. The SCENIHR Opinion, according to the Terms of Reference, is intended as Guidance on how to evaluate the risk when a nanomaterial is used in a medical device. The Opinion is not intended to clarify the status of borderline products. It is up to the regulators to decide on such borderline products and on classification issues in general.

The text of the comments received and the response provided by the SCENIHR are available here:

http://ec.europa.eu/health/scientific_committees/consultations/public_consultations/scenihr_consultation_22_en.htm

## 7. MINORITY OPINION

None

## 8. ABBREVIATIONS AND GLOSSARY OF TERMS

| Term | Explanation |
|---|---|
| AAMI | Association for the Advancement of Medical Instrumentation |
| AAS | Atomic Absorption Spectroscopy |
| ABPM | Ambulatory Blood Pressure Measurements |
| Absorption of energy | The way by which the energy of a photon, which is the quantum of the electromagnetic field, is taken up by matter, typically the electrons of an atom. |
| ADME | Absorption, Distribution, Metabolism, and Excretion (toxicokinetics) |
| AIMD | Active implantable medical device |
| AFM | Atomic-force microscopy |
| AUC | Analytical Ultracentrifugation |
| ARMD | Adverse Reaction to Metal Debris, overall description of local reactions (observed by histopathology) near metal on metal hip prostheses due to release of metal particles |
| BET | Specific surface area measurements; Brunauer–Emmett–Teller (BET) theory aims to explain the physical adsorption of gas molecules on a solid surface |
| CLS | Centrifugal liquid sedimentation |
| DLS | Dynamic Light Scattering is a method for measuring the particle size distribution in an ensemble |
| DMA | Differential mobility analysis |
| EDX | Energy dispersive X-ray allows analysis of particles down to nanometre diameters |
| EELS | Electron energy loss spectroscopy allows analysis of particles down to nanometre diameters |
| EFSA | European Food Safety Authority |
| EN 15051:2006 | Procedure for determination of inhalable dustiness (dustiness values, stated as the ratio of the weight of the amount of released dust to the amount of material charged). The standard describes two methods that are based on a British method (MDHS 81) and a presently withdrawn German method (DIN 33897:2). The two methods represent different systems for supplying the mechanical energy. |
| FESEM | Field Emission Scanning Electron Microscopy |
| FEGSEM | Variant of FESEM, with a gun emitter |
| FFF | Field-flow fractioning |
| FTIR | Fourier Transform Infrared Spectroscopy |

| | |
|---|---|
| Free nanomaterial | Nanomaterials that are not encapsulated or connected in some way to prevent them from being released in the organs, tissues or cells of the user |
| GC-MS | Gas chromatography–mass spectrometry: the sample is usually ionized directly or indirectly by an electron beam. The high-energy electrons cause the formation of free radical ions. |
| HDC | Hydrodynamic chromatography |
| HPLC | High-performance liquid chromatography |
| HRTEM | High Resolution Transmission Electron Microscopy |
| ICP-MS | Inductively Coupled Plasma - Mass Spectrometertry |
| LC-MS | Liquid chromatography – mass spectrometry |
| LDE | Laser Doppler Electrophoresis |
| LLNA | Local Lymph Node Assay, murine assay to evaluate potential of chemicals for induction of delayed type hypersensitivity. |
| MPI | Magnetic Particle Imaging |
| MPS | Mononuclear Phagocytic System |
| MS | Mass spectrometry |
| Nano-object | A material with one, two or three external dimensions on a nanoscale. Nano-objects with two external dimensions on the nanoscale and a larger third dimension include nanofibres, nanotubes, nanofilaments or nanorods. |
| Nano-particle | A nano-object with three external dimensions on a nanoscale |
| Nano-reinforced materials | Nano-objects included in their matrices to introduce a new function or to alter physical and mechanical properties. Nanocomposites are a typical case. |
| Nanoscale | Dimensions between 1 and 100 nanometers |
| Nano-structured material | A material with a surface or internal structure on a nanoscale and possessing one or more new physical, chemical and biological properties specific to the nanoscale. |
| NMR | Nuclear Magnetic Resonance |
| NTA | Nanoparticle Tracking Analysis |
| OECD | Organisation for Economic Co-operation and Development, Paris, France |
| PALS | Phase analysis light scattering (PALS configuration has been shown to be able to measure mobility at least two orders of magnitudes lower than conventional LDE) |
| PET | Positron emission tomography |
| PTA | Particle-tracking analysis is a counting method that study particle by particle |

| | |
|---|---|
| Redox potential | A measure of the tendency of a chemical species to acquire electrons and thereby be reduced |
| SAR | Structure Activity Relationship |
| SAXS | Small-Angle X-ray Scattering reports on intensity-weighted particle size; it is in the same class of methods as DLS |
| SCCS | Scientific Committee on Consumer Safety |
| SCENIHR | Scientific Committee on Emerging and Newly Identified Health Risks |
| SCHER | Scientific Committee on Health and Environmental Risks |
| SEC | Size-exclusion chromatography |
| SEM | Scanning Electron Microscopy |
| SERS | Surface enhanced Raman Spectroscopy |
| SIMS | Secondary Ion Mass Spectrometry |
| SMPS | Scanning Mobility Particle Size |
| SP-ICP-MS | Single particle inductively coupled plasma mass spectrometer |
| SPM | Suspended particulate matter |
| SPM | Scanning Probe Microscopy |
| STEM | Scanning Transmission Electron Microscopy. Offers an alternative configuration of TEM and an extended range of analytical methods. In the STEM, as in the SEM, a finely focused electron beam is scanned across a raster on the specimen. |
| STM | Scanning Tunnelling Microscopy |
| TDI | Tolerable Daily Intake |
| TEGDMA | Triethylene glycol dimethacrylate |
| TEM | Transmission Electron Microscopy |
| TNF | Tumour Necrosis Factor |
| US-EPA | United States Environmental Protection Agency |
| UV spectroscopy | Ultra-violet spectroscopy intended for chemical analysis |
| UVVis | Ultra-violet visible spectroscopy |
| XPS | X-ray photoelectron spectroscopy, also known as ESCA |
| X-ray absorption spectroscopy | A technique for determining the local geometric and/or electronic structure of matter |
| XRD | X-Ray Diffraction is a method for measurement of an |

average size value without giving information about the size distribution

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## Annex

### Performance of some Characterisation Methods

Several methods have been identified and each method has its own performance in terms of possibilities and limitations.

### Electron microscopy techniques

Electron microscopy is perhaps the most generally applicable method. Scanning Electron Microscopy (SEM) and transmission electron microscopy (TEM) are two types of electron microscopes and are tools to view and examine small samples. Both instruments use electrons or electron beams. The images produced in both tools are highly magnified and offer high resolution. SEM measures the shape and size of the particles, topography of the surface and determines the composition of elements and compounds the sample is composed of. In SEM the specimen surface is scanned with a high-energy electron beam and scattered electrons are measured while the TEM is based on transmitted electron measurements TEM seeks to see what is inside or beyond the surface. SEM also shows the sample bit by bit while TEM shows the sample as a whole. In terms of magnification and resolution, TEM has an advantage compared to SEM. TEM has up to a 50 million magnification level while SEM only offers 2 million as a maximum level of magnification. The resolution of TEM is 0.5 angstroms while SEM has 0.4 nanometers. However, SEM images have a better depth of field compared to TEM produced images. In SEM, the sample is prepared on specialised aluminium stubs and placed on the bottom of the chamber of the instrument. The image of the sample is projected onto the CRT or television-like screen. On the other hand, TEM requires the sample to be prepared in a TEM grid and placed in the middle of the specialised chamber of the microscope. The microscope via fluorescent screens produces the image. Another feature of SEM is that the area where the sample is placed can be rotated in different angles.

The scanning transmission electron microscope (STEM) offers an alternative configuration of transmission electron microscopy, and with it an extended range of analytical methods. In the STEM, as in the SEM, a finely focused electron beam is scanned across a raster on the specimen. Resultant signals used to image the specimen include the intensity of the transmitted beam, secondary electron emissions and elastically scattered electrons.

TEMs are usually configurable as STEMs, although there is inevitably a degree of compromise with the electron optics, resulting in marginally reduced imaging and analysis capabilities. Spatial resolution in a dedicated STEM is typically better than 1 nm, and may approach *ca*. 0.3 nm in a high-resolution system.

Size and morphology are readily characterised in the FEGSEM, TEM and STEM. HRTEM allows structural information on particles and atomic clusters to sub-0.2 nm resolution, while EELS and EDX analysis in the STEM allow the chemical analysis of particles down to nanometre diameters. By combining analysis methods, investigation of particle size, shape, structure, composition and surface properties is in principle possible.

However, the analysis environment is harsh, and only suited to robust particles with low volatility. Analysis in the ESEM overcomes some of the analysis environment restrictions and allows in principle the characterisation of particles with a significant volatile component, although its application is currently restricted to particles larger than *ca*. 100 nm.

The use of X-ray emissions within the electron microscope is perhaps the most widely applied form of analytical electron microscopy within aerosol. Electrons interacting with

the specimen excite inner shell atomic electrons, and the decay of these excited states leads to the emission of X-rays with energies characteristic of the element.

Energy dispersive X-ray analysis (EDX) allows the quantification of elemental species of atomic number 6 (carbon) and above in the SEM, ESEM, TEM and STEM, although many detectors using a thin silicon protective window are limited to the detection of elements of atomic number 14 (silicon) and above. Analysis in the SEM is not ideal for ultrafine particles, as X-ray emissions from the holding substrate rapidly obscure those from particles under analysis.

For the same reason, spatial resolution within the SEM is relatively low (of the order of 0.5 -1 micrometer). Spatial resolution in the STEM and TEM approaches the electron beam width when using thin substrates or arranging for samples to be over a hole on the substrate. Sensitivity to high Z elements is sufficient for the identification of major elemental species in nanometer-diameter particles.

The sensitivity of EDX analysis in the TEM and STEM is limited by the relatively low detection efficiency for X-ray emissions. However, each core electron excitation within the specimen results in a corresponding energy loss within the electron beam.

By extracting energy loss information from the beam using an energy-dispersive spectrometer, increased sensitivity to core electron excitations is achievable. Electron energy loss spectroscopy (EELS) within the STEM (and TEM in some configurations) is perhaps the most powerful analysis technique available for analysing single particles within the electron microscope.

By recording and analysing the electron energy loss spectrum, details of specific inelastic interactions, and thus sample composition and structure, can be investigated. Energy losses below 50 - 100 eV are dominated by bulk electron excitations (plasmons) within the sample. At higher-energy losses, energy loss is characterised by atomic core electron excitations, appearing as `edges' on a decreasing background. The position, amplitude and shape of each edge contain information on atomic core electron excitations, and the chemical environment surrounding the atom. The energy loss at which the edge occurs is related to the atomic electron transition, allowing identification of elemental components

**Scanning probe microscopy (SPM) and Scanning Tunneling Microscopy (STM)**
The development of SPM methods has led to further techniques for imaging nanometer-sized particles. All methods are typified by a fine probe that is scanned in a raster across a surface. Probe position above (or on) the surface is controlled by a range of feedback signals which are also used to provide image contrast on the associated display raster.

Initial SPM development used the electron tunneling current between a conducting specimen and probe suspended a few angstroms above its surface to map topographic features at angstrom resolution (scanning tunneling microscopy (STM).

**Atomic Force Microscopy**
Later developments led to the use of Van der Waals forces between the specimen and the probe (atomic force microscopy (AFM)), allowing imaging of non-conducting specimens. While a gap of *ca*. 1nm is maintained between the probe and specimen in STM, AFM may be carried out with the probe in contact with the specimen, or separated by up to several tens of angstroms. AFM can measure topology, grain size, frictional characteristics and different forces. It consists of a silicon cantilever with a sharp tip with a radius of curvature of a few nanometers. The tip is used as a probe on the specimen to be measured. The forces acting at the atomic level between the tip and the surface of the specimen cause the tip to deflect and this deflection is detected using a laser spot which is reflected to an array of photodiodes. AFM has several advantages over the scanning electron microscope (SEM). Unlike the electron microscope, which provides a two-

dimensional projection or a two-dimensional image of a sample, the AFM provides a three-dimensional surface profile. Additionally, samples viewed by AFM do not require any special treatments (such as metal/carbon coatings) that would irreversibly change or damage the sample, and does not typically suffer from charging artifacts in the final image. While an electron microscope needs an expensive vacuum environment for proper operation, most AFM modes can work perfectly well in ambient air or even a liquid environment. This makes it possible to study biological macromolecules and even living organisms. In principle, AFM can provide higher resolution than SEM. It has been shown to give true atomic resolution in ultra-high vacuum (UHV) and, more recently, in liquid environments. High resolution AFM is comparable in resolution to scanning tunneling microscopy and transmission electron microscopy. AFM can also be combined with a variety of optical microscopy techniques, further expanding its applicability. Combined AFM-optical instruments have been applied primarily in the biological sciences but have also found a niche in some materials applications, especially those involving photovoltaics research {Ref. Geisse, Nicholas A. (July–August 2009). "AFM and Combined Optical Techniques". Materials Today 12 (7-8): 40–45. doi:10.1016/S1369-7021(09)70201-9}. A disadvantage of AFM compared with the scanning electron microscope (SEM) is the single scan image size. In one pass, the SEM can image an area on the order of square millimeters with a depth of field on the order of millimeters, whereas the AFM can only image a maximum height on the order of 10-20 micrometers and a maximum scanning area of about 150×150 micrometers. The scanned area size for AFM can be improved by using parallel probes in a fashion similar to that of millipede data storage {Ref. R. V. Lapshin (2007). "Automatic drift elimination in probe microscope images based on techniques of counter-scanning and topography feature recognition" (PDF). Measurement Science and Technology (UK: IOP) 18 (3): 907–927. Bibcode 2007MeScT..18..907L. doi:10.1088/0957-0233/18/3/046. ISSN 0957-0233}.

The scanning speed of an AFM is also a limitation. Traditionally, an AFM cannot scan images as fast as a SEM, requiring several minutes for a typical scan, while a SEM is capable of scanning at near real-time, although at relatively low quality. The relatively slow rate of scanning during AFM imaging often leads to thermal drift in the image

**Other Scanning Probe Microscopy (SPM) techniques**

The use of further feedback mechanisms has led to a number of SPM imaging methods, including magnetic force microscopy, lateral force microscopy, shear force microscopy and near field scanning optical microscopy. All methods can be operated in a range of environments, including atmospheric conditions, liquid immersion and vacuum Scanning Tunneling Microscopy (STM), which measures the 3-D topology of the specimen, is based on the concept of quantum tunneling. Electrons from the specimen can tunnel through the vacuum between the conducting tip and the surface in interest due to voltage difference between the tip and the surface. Monitoring the current as the tip's position scans across the surface, which can then be used to display an image, makes measurements.

SPM offers the possibility of analysing nanometre-diameter particles under ambient conditions, thus getting away from some of the constraints imposed by electron microscopy. Imaging methods such as AFM and NSOM offer novel and exciting possibilities for the characterisation of specific aerosols. For instance, the use of NSOM to identify, size and count fluorescently tagged ultrafine particles would seem applicable to identifying particle transport and deposition characteristics within biological systems. While SPM is currently limited in the information that can be obtained from ultrafine aerosol samples, the uniqueness of the information available should allow it to be developed as a complementary tool to electron microscopy.

While electron microscopy and SPM are confined to the analysis of collected samples and are constrained by the limitations of the collection and preparation systems used, developments in aerosol mass spectrometry are providing the means for chemically characterizing size-segregated ultrafine particles on-line.

Current technology allows the speciation of individual particles *ca*. 10 nm in diameter, and as this is reduced still further, the resulting methods should provide invaluable complementary data to off-line methods.
By adopting technologies developed within complementary disciplines, together with the development of aerosol-specific methods, it is possible to develop a basis for characterizing single sub-100 nm particles and features in terms of size, morphology topology, composition, structure and physicochemical properties.

The available methods provide complementary means to characterise single ambient particles in depth. Currently, with few exceptions, they are complex, time-consuming to use, and in many cases still at a developmental stage. As such they are not ideally suited to the routine analysis of aerosols. However, by adopting a multi-disciplinary approach, the potential is there to develop complementary tools that will provide routine and detailed information on the particles that influence the environment we live and work in.

**Small-angle X-ray scattering (SAXS)**
Small-angle X-ray scattering (SAXS) is a small-angle scattering (SAS) technique where the elastic scattering of X-rays (wavelength 0.1 ... 0.2 nm) by a sample which has inhomogeneities in the nm-range, is recorded at very low angles (typically 0.1 - 10°). This angular range contains information about the shape and size of macromolecules, characteristic distances of partially ordered materials, pore sizes, and other data. SAXS is capable of delivering structural information of macromolecules between 5 and 25 nm, of repeat distances in partially ordered systems of up to 150 nm {Ref. Glatter O, Kratky O, ed. (1982). Small Angle X-ray Scattering. Academic Press. ISBN 0-12-286280-5. } USAXS (ultra-small angle X-ray scattering) can resolve even larger dimensions. SAXS and USAXS belong to a family of X-ray scattering techniques that are used in the characterisation of materials. In the case of biological macromolecules such as proteins, the advantage of SAXS over crystallography is that a crystalline sample is not needed. Nuclear magnetic resonance spectroscopy methods encounter problems with macromolecules of higher molecular mass (> 30-40 kDa). However, owing to the random orientation of dissolved or partially ordered molecules, the spatial averaging leads to a loss of information in SAXS compared to crystallography. The P(r) function or pair-distance distribution function describes the paired-set of all distances between points within an object. In SAXS, the P(r) function is used to describe the paired-set of distances between all of the electrons within the macromolecular structure and is a useful tool for visibly detecting conformational changes within a macromolecule. Since the function describes the set of all paired-distances within a structure, small changes in the relative positions of a few residues can result in detectable changes in a P(r) distribution.

**Light scattering techniques**
Dynamic light scattering (DLS) (also known as photon correlation spectroscopy or quasi-elastic light scattering) is a technique in physics that can be used to determine the size distribution profile of small particles in suspension or polymers in solution {Ref. It can also be used to probe the behavior of complex fluids such as concentrated polymer solutions.

NanoSight have developed a unique instrument, which allows the tracking of the Brownian motion of nanoparticles in liquid suspension on a particle-by-particle basis. Subsequent application of the Stokes-Einstein equation allows the determination of particle size. Particle count is also available. This technique presents a powerful alternative to more typical light scattering techniques such as DLS for the analysis of complex and polydisperse sample types of varying composition. Both DLS and nanoparticle tracking analysis (NTA) measure the Brownian motion of nanoparticles whose speed of motion, or diffusion coefficient, is related to particle size through the Stokes-Einstein equation. NTA provides linear size axes, a high-resolution scale compared to wide logarithmic scale in DLS, particle concentration information on the

vertical axis. Standard polystyrene beads of sizes ranging from 60 to 1,000 nm and physical mixtures thereof were analyzed with NTA and DLS. The influence of different ratios of particle populations was tested. Drug delivery nanoparticles and protein aggregates were analyzed by NTA and DLS. Also live monitoring of heat-induced protein aggregation was performed with NTA.